REMARKS

I. Amendments to the Claims

Claims 1-22, 24, 32, 41-58 and 63-70 have been canceled without prejudice. Applicant reserves the right to prosecute the subject matter of any canceled claims in one or more continuation, continuation-in-part, or divisional applications.

Claim 23 has been amended to clearly define the subject matter of the invention by deleting 'formation or', changing 'tumors' to 'blood-born tumors' and changing 'an effective amount' to 'a therapeutically effective amount', as proposed at a personal interview held on October 9, 2007. The claim is supported by the originally filed specification, for example, page 5, lines 12-19, page 9, lines 7-9, page 20, lines 15-24 and canceled claim 58. Claim 34 has been amended to remove tumors other than blood-born tumors. Claim 71 has been amended to correct claim dependency. Claim 72 has been added to recite a capsule. The claim is supported by the originally filed specification, for example, page 21, lines 2-9 and claim 26. No new matter has been added.

Claims 23, 25-31, 33-40, 59-62 and 71-72 are pending. Applicant respectfully submits that the pending claims are allowable for the following reasons.

II. Applicant's Statement of the Substance of Interview and Response to the Examiner's Interview Summary of Record

A personal interview with Patent Examiner Anderson, Dr. Schafer, Dr. Robertson-Chow, Mr. Girards and Ms. Moon, attorneys for Applicant, was held on October 9, 2007. Applicant appreciates the Examiner interview.

During the interview, the Examiner and attorneys for Applicant discussed the pending § 112 enablement rejections. Attorneys for Applicant pointed out that the pending claims are enabled based on the Examples and descriptions in the specification. Attorneys for Applicant presented articles published after the filing date of the application as evidence that a skilled person in the art can practice the claimed invention without undue experimentation based upon the disclosure of the specification. The articles report that inhibition of angiogenesis by thalidomide was discovered by the present inventor, and that thalidomide is now used for treating tumors, especially blood-born tumors (*e.g.*, multiple myeloma) in the United States. Attorneys for Applicant also presented a label of thalidomide which was approved to treat multiple myeloma by the U.S. Food and Drug Administration in June 2006.

Attorneys for Applicant proposed the amendments of the claims to recite the inhibition of the growth of blood-born tumors. The Examiner stated that he and his supervisor would very

favorably consider such amendment and evidence. Attorneys for Applicant noted that they would submit an amendment to the claims as proposed. Applicant's arguments and evidence are discussed below and presented herewith.

III. Arguments and Response to Rejections

The Claimed Invention is Definite

Claims 23, 25-26, 30-31, 33-35, 39-43, 47-52 and 56-71 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite, because 'an effective amount' recited in the claims is not clear what the amount being administered is effective for. (Page 8 of Office Action). Applicant respectfully traverses the rejection.

Solely to promote the allowance of the case and without acquiescing to the Examiner's rejection, claim 25 has been amended by changing 'an effective amount' to 'a therapeutically effective amount', as proposed at the interview. The specification, for example on page 20, clearly describes the therapeutically effective amount for inhibiting the growth of tumors and treating tumors. Therefore, Applicant respectfully requests that the rejection of the claims under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

The Claimed Invention Meets Enablement Requirements

Claims 23, 25-31 and 33-71 are rejected under 35 U.S.C. §112 as failing to comply with the enablement requirement. (Pages 3-7 and 9-18 of Office Action). Applicant respectfully traverses the rejection.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *U.S. v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). As to the meaning of undue experimentation, the Office Action states that "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a <u>reasonable amount of guidance</u> with respect to the direction in which experimentation should proceed to enable the determination of how to practice a desired embodiment of the claimed invention." (Page 9 of the Office Action). (emphasis added). Applicant respectfully submits that the pending claims, particularly as amended, comply with the enablement requirement, because the specification provides a reasonable amount of guidance to practice the claimed invention, as discussed below.

In this response, claim 23 has been amended to recite "a method for inhibiting the growth of blood-born tumors sensitive to thalidomide in a human comprising administering to said human a therapeutically effective amount of thalidomide." Thus, the pending claims encompass

methods using thalidomide for inhibiting the growth of blood-born tumors sensitive to thalidomide. In fact, the Examiner at the interview stated that the amendment of the claims to recite inhibiting the growth of blood-born tumors and evidence on the efficacy of thalidomide in tumors, especially blood-born tumors, would overcome the enablement rejection.

Specifically, the specification discloses the methods of administering thalidomide to patients having tumors, including mode of administration, dosage forms and formulations (*e.g.*, page 20, lines 2-8, and page 20, line 25 to page 23, line 2, for claims 23, 25, 26, 31, 35, 39, 40, 59-62 and 71), and doses of thalidomide (*e.g.*, page 20, lines 15-24 for claims 27-29 and 36-38). Thus, one skilled in the art would have been able to practice the claimed invention by administering the specified amount of thalidomide using the specified routes of administration to the specified patients, in accordance with the explicit teachings of the present application.

The specification also discloses working Examples I through III (pages 24-28, chick chorioallantoic membrane (CAM) assay, rabbit cornea angiogenesis assay and inhibition of bFGF induced corneal neovascularization), demonstrating that thalidomide is effective in inhibiting angiogenesis *in vivo*. The inhibition of angiogenesis by thalidomide is described on page 27 of the specification, and Figures 6 and 7. The specification clearly describes that undesired angiogenesis occurs in tumors and the inhibition of undesired angiogenesis halts tumor growth. (*e.g.*, page 2, lines 19-26; page 4, lines 20-27 and page 5, line 1 to page 6, line 3). The Office Action has also recognized that undesired angiogenesis is associated with tumor growth and it has not questioned that thalidomide inhibits angiogenesis. (Page 4 of the Office Action).

Thus, from the description of the specification, one skilled in the art would have been able to appreciate that the inhibition of angiogenesis by administration of thalidomide would lead to the inhibition of the growth of tumors. In view of the foregoing, the specification provides a sufficient guidance as to inhibiting the growth of tumors, by administering an effective amount of thalidomide.

Nonetheless, the Office Action states that the articles cited by the Examiner disclose ineffectiveness of thalidomide against cancer, and thus they support lack of enablement of the claimed invention. (Office Action, pages 12-15). Applicant points out that the present application is distinct from the articles published prior to the filing date of this application, because the specification- unlike the articles published prior to the filing date of this application- discloses sufficient guidance to practice the claimed invention for inhibiting the growth of tumors using thalidomide without undue experimentation.

Applicant notes that the references of record (published prior to the filing date of the instant application) disclosed ineffectiveness of thalidomide against cancer in certain studies[1]. Because of the disappointing results of the study reports, interest in thalidomide as an anti-cancer agent diminished greatly. *See, e.g.*, previously submitted Diggle, page 630, left column, the second paragraph. Since then, however, the study of the present inventor D'Amato on anti-angiogenesis - related to the invention disclosed in the present application - prompted the numerous trials on use of thalidomide in cancer. *See, e.g.*, Diggle, page 630, left column, the second paragraph; Kumar, page 2478, the second paragraph; and Rajkumar, page 900, the last two paragraphs.

Indeed, there was an explosion of studies for thalidomide in cancer in the late 1990's and, it is believed that this was in part, in response to the disclosures made by D'Amato in the present specification. In the last response, Applicant submitted articles which reported that thalidomide had been being studied and used for treating tumors, based on the discovery of the present inventor that thalidomide inhibits angiogenesis. *See, e.g.*, previously submitted Diggle, Kumar and Rajkumar. The article of the present inventor D'Amato study (*Proc. Natl. Acad. Sci.*, USA 91, 1994, page 4082-5) also describes that thalidomide was effective in inhibiting angiogenesis *in vivo* in rabbit cornea angiogenesis assay, and concludes that there are clear implications for the use of this drug for treating angiogenesis associated diseases including tumors. *See*, at page 4085.

In addition to the articles previously submitted , the following articles also evidence that a skilled in the art can use and practice the claimed invention from the disclosure of the specification. For example, Singhal *et al.* (Antitumor Activity of Thalidomide in Refractory Multiple Myeloma, *N. Engl. J. Med.*, 1999, pp. 1565-1571) describes that <u>angiogenesis is important in tumor progression, that thalidomide can inhibit angiogenesis in animal models, and that angiogenesis-inhibiting drug thalidomide may be useful for treating cancers</u> (page 1565, 2nd paragraph of right column). A copy of the article was submitted at the interview and is submitted herewith again. Indeed, the author referred to the present inventor's animal model studies of angiogenesis, which are described in the instant specification, describing that thalidomide has been shown to inhibit angiogenesis induced by fibroblast growth factor and vascular endothelial growth factor in a rabbit-cornea micropocket assay and a murine model of corneal vascularization, and also to cause apoptosis of established tumor-associated angiogenesis in experimental model (page 1570, left column and page 1571).

[1] Applicant makes no admission that such references are enabling, nor any admission as to the contents of their disclosure by this statement.

Kneller A. et al. (Therapy with thalidomide in refractory multiple myeloma patients - the revival of an old drug. *British Journal of Haematology* 2000; 108(2): 391-3) also reports that angiogenesis plays important role in haematological malignancies; that thalidomide has an inhibitory effect on angiogenesis as shown in an animal model by the present inventor D'Amato; and that this could be beneficial action in treating multiple myeloma (page 393, left column).

Hideshima T. et al. (Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* 2000; 96(9): 2943-50) further reports that the discovery that thalidomide possessed potent antiangiogenic properties by D'Amato provided rationale for its use to treat multiple myeloma (page 2943, left column).

Barlogie B. et al. (Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients. *Blood* 2001; 98(2): 492-4) also reports on page 492, left column that the present inventor D'Amato demonstrated the anti- angiogenic effects of thalidomide by repression of vascular endothelial growth factor and basic fibroblast growth factor pathways, and concludes on page 493, right column that the activity of thalidomide in multiple myeloma involving an antiangiogenic mechanism led people in the field to investigate antiangiogenic agents for antitumor activity.

Thus, the publications confirm that it was the breakthrough made by the present inventor which demonstrated that thalidomide inhibits angiogenesis, could be used in inhibiting tumors and re-ignited interest in the drug, and thalidomide is now being used and studied further as a treatment of tumor such as multiple myeloma. This art is evidence that a skilled person in the art can practice the claimed invention without undue experimentation based upon the disclosure of the specification.

Next, the Office Action states the disclosure of the specification that thalidomide inhibits angiogenesis does not reasonably suggest that it will be effective in inhibiting tumor growth in humans. (Page 16 of the Office Action). Applicant respectfully disagrees.

The specification clearly describes the relationship between the inhibition of angiogenesis and the inhibition of tumor growth in humans. For example, the specification at page 2, lines 19-26; page 4, lines 20-27 and page 5, line 1 to page 6, line 3, states as follows:

> "Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped together as angiogenic dependent or angiogenic associated diseases. Therapies directed at control of the angiogenic processes

could lead to the abrogation or mitigation of these diseases....

One of the most frequent angiogenic diseases of childhood is the hemangioma.....

Angiogenesis is prominent in solid tumor formation and metastasis. Angiogenic factors have been found associated with several solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is important include solid tumors, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas. Prevention of angiogenesis could halt the growth of these tumors and the resultant damage to the animal due to the presence of the tumor.

It should be noted that angiogenesis has been associated with **blood-born tumors** such as **leukemias**, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and spleen. It is believed that angiogenesis plays a role in the **abnormalities in the bone marrow** that give rise to **leukemia-like tumors**.

Angiogenesis is important in two stages of tumor metastasis. The first stage where angiogenesis stimulation is important is in the vascularization of the tumor which allows tumor cells to enter the blood stream and to circulate throughout the body. After the tumor cells have left the primary site, and have settled into the secondary, metastasis site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis could lead to the prevention of metastasis of tumors and possibly contain the neoplastic growth at the primary site.

Knowledge of the role of angiogenesis in the maintenance and metastasis of tumors has led to a prognostic indicator for breast cancer....Control of angiogenesis by therapeutic means could possibly lead to cessation of the recurrence of the tumors."

Thus, the specification clearly describes that the inhibition of angiogenesis would lead to the inhibition of the growth of tumors such as blood born-tumors in humans.

In addition, in the specification at pages 24-28 of the present application, Examples I to III (chick chorioallantoic membrane (CAM) assay, rabbit cornea angiogenesis assay and inhibition of bFGF induced corneal neovascularization) demonstrate that thalidomide is effective in inhibiting angiogenesis *in vivo*. In particular, the specification on page 27 discloses that treatment with a dose of (200mg/kg) of thalidomide resulted in an inhibition of the area of vasularized cornea that ranged from 30-51% in the rabbit cornea angiogenesis assay, with a

median inhibition of 36% (Figure 6) (n=30 eyes, p=0.0001), and Figure 7 shows the inhibition of angiogenesis by thalidomide. Thus, the specification describes that the inhibition of angiogenesis by thalidomide resulting from the assays in the examples would lead to the inhibition of the growth of tumors such as blood born-tumors in humans.

Accordingly, the specification and references of record supports the use of the assay described in the specification as an indicator of activity against angiogenesis and tumors such as blood born-tumors in humans. Where a particular model is recognized as correlating to a specific condition in a given art, the Examiner should accept that correlation, unless the Examiner has evidence that the model does not correlate. *In re Brana* at 1566; MPEP. § 2164.02. The Office Action has not provided with any evidence that the animal model disclosed in the specification does not correlate with the inhibition of tumors in humans.

Further, Applicant respectfully submits that an *in vitro* or *in vivo* animal model examples in the specification, in effect, constitute working examples, as the examples correlate with a claimed method invention. *In re Brana*, at 1566; MPEP § 2164.02. "A rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence." *See Cross v. Iizuka*, 753 F.2d 1040, 1050 (Fed. Cir. 1985). With this legal framework in mind, Applicant submitted publications to show the correlations between the methods for inhibition of the growth of tumors in humans and the animal models for angiogenesis inhibition described in the specification. *See e.g.*, Langer *et al.*, Gimbrone *et al.*, "Tumor Growth and Neovascularization: An Experimental Model Using the Rabbit Cornea," *J. Natl. Cancer Inst.* (1974) 52(2): 413-427, and D'Amato *et al.*, "Thalidomide is an Inhibitor of Angiogenesis," *Proc. Natl. Acad. Sci., U.S.A.* (1994) 91: 4082-4085.

Applicant notes that human working examples are not required under 35 U.S.C., first paragraph. For example, in *In re Brana*, the PTO alleged that animal testing was not reasonably predictive of the success of the claimed compounds for treating cancer in humans. 51 F.2d 1560, 1567, 34 U.S.P.Q.2d 1436 (Fed Cir. 1995). The Court rejected this argument and stated that "[t]he Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption." *Id.* The Court further stated that "<u>Title 35 does not demand that such human testing occur within the confines of the Patent and Trademark Office Proceedings</u>." *Id.*, citing *Scott v. Finney*, 34 F.3d 1058, 1063, 32 U.S.P.Q.2d (BNA) 115, 120 (Fed. Cir. 1994) (emphasis added). Thus, Applicant respectfully submits that Applicant is not required to provide any clinical data of the efficacy and safety of thalidomide.

In view of the clear descriptions of the relationship between the inhibition of

angiogenesis and tumors in humans in the specification, one skilled in the art would have been able to practice the claimed invention by administering the specified amount of thalidomide using the specified routes of administration to patients having tumors.

In sum, Applicant respectfully submits that the specification provides sufficient information and guidance to those of ordinary skill in the art to make and use the claimed invention, and that to the extent any experimentation is necessary, such experimentation is not undue. Therefore, Applicant respectfully requests that the rejection of the claims under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

The Double Patenting Rejection Should Be Withdrawn

Claims 23, 25-29, 31, 33-40, 58-62, 67-68 and 71 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 25-46 of copending Application No. 11/096,155. (Office Action, pages 18-19).

Solely to promote the allowance of the case and without acquiescing to the Examiner's rejection, a terminal disclaimer was submitted in Application No. 11/096,155 on October 30, 2007. Thus, Applicant respectfully requests that the double patenting rejections be withdrawn.

IV. Conclusion

Applicant respectfully requests that the above amendments and remarks be entered in the file of this application. Should the Examiner not agree that all claims are allowable, then a further personal or telephonic interview is respectfully requested to discuss any remaining issues and to accelerate the allowance of the above-identified application. Please charge any required fees to Jones Day Deposit Account No. 50-3013.

Respectfully submitted,

Date: October 31, 2007

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ANTITUMOR ACTIVITY OF THALIDOMIDE IN REFRACTORY MULTIPLE MYELOMA

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ABSTRACT

Background Patients with myeloma who relapse after high-dose chemotherapy have few therapeutic options. Since increased bone marrow vascularity imparts a poor prognosis in myeloma, we evaluated the efficacy of thalidomide, which has antiangiogenic properties, in patients with refractory disease.

Methods Eighty-four previously treated patients with refractory myeloma (76 with a relapse after high-dose chemotherapy) received oral thalidomide as a single agent for a median of 80 days (range, 2 to 465). The starting dose was 200 mg daily, and the dose was increased by 200 mg every two weeks until it reached 800 mg per day. Response was assessed on the basis of a reduction of the myeloma protein in serum or Bence Jones protein in urine that lasted for at least six weeks.

Results The serum or urine levels of paraprotein were reduced by at least 90 percent in eight patients (two had a complete remission), at least 75 percent in six patients, at least 50 percent in seven patients, and at least 25 percent in six patients, for a total rate of response of 32 percent. Reductions in the paraprotein levels were apparent within two months in 78 percent of the patients with a response and were associated with decreased numbers of plasma cells in bone marrow and increased hemoglobin levels. The microvascular density of bone marrow did not change significantly in patients with a response. At least one third of the patients had mild or moderate constipation, weakness or fatigue, or somnolence. More severe adverse effects were infrequent (occurring in less than 10 percent of patients), and hematologic effects were rare. As of the most recent follow-up, 36 patients had died (30 with no response and 6 with a response). After 12 months of follow-up, Kaplan–Meier estimates of the mean (±SE) rates of event-free survival and overall survival for all patients were 22±5 percent and 58±5 percent, respectively.

Conclusions Thalidomide is active against advanced myeloma. It can induce marked and durable responses in some patients with multiple myeloma, including those who relapse after high-dose chemotherapy. (N Engl J Med 1999;341:1565-71.)

MULTIPLE myeloma accounts for approximately 1 percent of all cancers and 10 percent of hematologic cancers. It is incurable with conventional chemotherapy.[1] Melphalan-based high-dose chemotherapy with hematopoietic stem-cell support increases the rate of complete remission and extends event-free and overall survival.[2-4] However, many patients still relapse, and options for salvage therapy are limited.[5,6]

Angiogenesis is important in embryogenesis, wound healing, diabetic retinopathy, and tumor progression.[7,8] The immunomodulatory drug thalidomide can inhibit angiogenesis and induce apoptosis of established neovasculature in experimental models.[9,10] For these reasons, angiogenesis-inhibiting drugs such as thalidomide may be useful for treating cancers that depend on neovascularization.

Prominent bone marrow vascularization occurs in multiple myeloma. It correlates positively with a high plasma-cell–labeling index (a poor prognostic sign) and disease activity and independently confers a poor prognosis.[11-16] Plasma levels of various angiogenic cytokines, such as basic fibroblast growth factor and vascular endothelial growth factor, are elevated in patients with active myeloma.[11-13,16] In 1965, Olson et al. reported slowing of disease progression in one patient who was treated with thalidomide.[17] These considerations led us to administer thalidomide to five patients with end-stage myeloma through a compassionate-use protocol. One patient with a large tumor burden (as indicated by an IgA level of 8.4 g per deciliter, the presence of more than 95 percent plasma cells in bone marrow, and the need for transfusion), who had had no response to two cycles of high-dose chemotherapy followed by multiple salvage therapies, had a nearly complete remission within three months after the initiation of thalidomide therapy. This observation prompted a phase 2 investigation of tha-

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lidomide in patients with advanced and refractory myeloma.

METHODS

Patients and Treatments

Between December 1997 and June 1998, 84 consecutive, eligible patients with previously treated and progressive myeloma began treatment with oral thalidomide as a single agent after providing written informed consent. No patients were excluded on the basis of renal or cardiopulmonary function, whereas patients could be excluded if the results of liver-function tests were more than twice the upper limit of normal levels. All patients were treated at a single center according to a phase 2 protocol approved by the institutional review board and the Food and Drug Administration (FDA).

Thalidomide was supplied in 50-mg capsules by Celgene (Warren, N.J.) and was administered nightly at a dose of 200 mg. The dose was increased by 200 mg every two weeks for six weeks, so that the final dose was 800 mg per day. Data were analyzed as of June 17, 1999, when the duration of treatment ranged from 2 to 465 days (median, 80) and the median follow-up of surviving patients was 13 months.

Table 1 summarizes the characteristics of the patients and details of prior therapy. Seventy-six patients (90 percent) had received at least one cycle of high-dose chemotherapy with autologous hematopoietic stem-cell support, and 58 (69 percent) had received two or more cycles of intensive chemotherapy. The median time from the last course of high-dose chemotherapy to the beginning of treatment with thalidomide was 14 months. A high-risk cytogenetic abnormality (deletion of chromosome 13) was present in 35 patients (42 percent).[20] One patient had received an allograft as a second intervention, with evidence of full donor-type chimerism in normal lymphohematopoietic cells. At the time of enrollment, all patients had progressive disease, with an increase in paraprotein levels of at least 25 percent or at least 50 percent plasma cells in bone marrow. Approximately half the patients had been retreated with dexamethasone or other regimens, but the disease had progressed before thalidomide treatment was begun.

Evaluation

The pretreatment evaluation included complete blood counts, tests of renal and liver function, serum and urine protein electrophoresis, and measurements of serum levels of immunoglobulins, $beta_2$-microglobulin, and C-reactive protein. Bone marrow aspirates were obtained and biopsies were performed to determine the percentage of plasma cells in bone marrow, to identify karyotypic abnormalities (Giemsa-banded cells in metaphase), and to assess the proliferative activity in plasma cells according to the bromodeoxyuridine method to derive the plasma-cell–labeling index.[18] Follow-up studies included a weekly estimation of paraprotein levels — the myeloma protein in serum and Bence Jones protein in urine — for the first two months, followed thereafter by monthly measurements. Whenever possible, bone marrow was examined at the time of the maximal response or when patients with no response left the study.

The microvascularity of bone marrow was studied in a semiquantitative fashion in biopsy samples that were obtained with a trephine and stained with an anti-CD34 monoclonal antibody (prediluted Clone QBEnd/10, Cell Marque, Austin, Tex.). The results were expressed as the number of vessels per high-power field (400×).

Assessment of Response

The primary end point of the study was the finding of a decline in the level of paraprotein in serum or urine of at least 25 percent, 50 percent, 75 percent, or 90 percent on two occasions at least six weeks apart. Among patients with detectable levels of both urine and serum paraprotein, the response was judged on the basis of the component showing the smaller decline. Patients with a reduction of less than 25 percent and those who discontinued treatment before a response could be assessed were considered to have had no response to thalidomide. Thus, the results were evaluated on an intention-to-treat basis. In patients with a response, an increase in serum or urine paraprotein levels by more than 25 percent above the nadir value was considered evidence of relapse. In patients who had a complete remission, evidence of reemergence of the monoclonal protein (determined by immunofixation) on at least two occasions was considered to indicate a relapse. In patients who had a complete remission or a nearly complete remission (≥90 percent reduction in serum or urine paraprotein levels), a bone marrow response was defined as the finding of less than 5 percent plasma cells in the biopsy specimen or aspirate. For the remaining patients with a paraprotein response, the percentage of plasma cells had to decrease by at least 50 percent to qualify as a bone marrow response.

Assessment of Adverse Effects

All patients, irrespective of the duration of therapy, were included in the evaluation of adverse effects. All patients received diaries after providing informed consent, and 83 patients (99 per-

Table 1. Characteristics of the Patients.

Characteristic	No. of Patients (%)
Male sex	61 (73)
Durie–Salmon stage III multiple myeloma	51 (61)
IgG paraprotein	51 (61)
Duration of prior therapy >60 mo	18 (21)
Prior high-dose chemotherapy	76 (90)
Receipt of >1 cycle of high-dose chemotherapy	58 (69)
Interval between last cycle of high-dose chemotherapy and initiation of thalidomide >12 mo	43 (57)
Age >60 yr	32 (38)
Hemoglobin <9 g/dl	19 (23)
Platelet count $<50\times10^3$/mm^3	17 (20)
Serum albumin <3.5 g/dl	22 (26)
Serum creatinine >1.5 mg/dl (133 μmol/liter)	22 (26)
Serum $beta_2$-microglobulin >6 mg/liter	24 (29)
Serum C-reactive protein >3 mg/liter	20 (24)
Serum monoclonal immunoglobulin >1 g/dl	51 (61)
Urine Bence Jones protein >1 g/day	44 (52)
>50% Plasma cells in bone marrow on biopsy	18 (21)
Plasma-cell–labeling index >1%*	13 (15)
Bartl grade II†	19 (23)
Deletion of chromosome 13	35 (42)
Outcome	
Completion of study	19 (23)
Withdrawal from study	
Progression	54 (64)
Intolerance of thalidomide	9 (11)
Death of patient with a response‡	1 (1)
Personal reasons	1 (1)
Final dose of thalidomide	
400 mg/day	72 (86)
600 mg/day	57 (68)
800 mg/day	46 (55)

*The plasma-cell–labeling index represents the percentage of light-chain–restricted plasma cells incorporating bromodeoxyuridine.[18]

†The Bartl grading system distinguishes myeloma cells according to their morphologic maturation.[19] Grade II refers to immature plasma cells of cleaved, asynchronous, or polymorphous appearance.

‡This patient had a response to treatment but died on day 37 of treatment.

cent) reported having adverse effects. A comprehensive checklist of the adverse effects associated with thalidomide therapy was provided by Celgene; it was based on previous experience in treating patients with leprosy and had been reviewed by the FDA. The data were verified by the patients by direct or telephone interviews. Hematologic values and other laboratory-based measures of adverse effects were assessed at least monthly by the data-management office.

Statistical Analysis

The primary end point for this phase 2 study was a diminution in the plasma level of the myeloma protein or the urine level of Bence Jones protein. Other end points included the time to a response, the time to disease progression, event-free survival, overall survival, the microvascularity of bone marrow, and improvements in other laboratory values. Response was treated as a categorical variable. Comparisons of the response according to other categorical variables were assessed with use of the chi-square test or Fisher's exact test, as appropriate. The times to response and disease progression were calculated with the use of the competing-risk methods.[21] The time to response was defined as the interval between the start of therapy and a given response (i.e., a decline in the serum or urine level of paraprotein of at least 25 percent, 50 percent, 75 percent, or 90 percent or a complete remission). Competing risks with respect to the time to response included discontinuation of treatment because of progression or a lack of response, an inability to tolerate thalidomide, or death or personal reasons. The time to progression was calculated only for patients with a paraprotein response and was defined as the time from the start of therapy to disease progression. Competing risks with respect to the time to progression included discontinuation of treatment because of adverse effects or death or for personal reasons. Event-free survival and overall survival were estimated according to the method of Kaplan and Meier.[22] Event-free survival was calculated from the start of therapy to disease progression, removal from the study for any reason, death from any cause, or the last follow-up visit, whichever occurred first. Overall survival was calculated from the start of therapy to death from any cause or the last follow-up visit. Data on patients who had not had an event by the time of the last follow-up were censored at that time with respect to times to response and progression, event-free survival, and overall survival. Survival was compared with use of the log-rank test.[23] Univariate and multivariate (stepwise) logistic-regression methods were used to evaluate the prognostic importance of various characteristics with respect to the likelihood of achieving at least a 25 percent or 50 percent reduction in serum or urine paraprotein levels. Univariate and multivariate (stepwise) proportional-hazards regression analyses were used to evaluate the prognostic importance of various characteristics with respect to event-free survival and overall survival.

Since the microvascular density of bone marrow was used as a measure of the antiangiogenic action of thalidomide, this variable was extensively modeled. To account for the need for multiple measurements of each patient over time and missing data, we used mixed-models repeated-measures analysis of variance to evaluate the microvascular density of bone marrow.[24] The use of compound symmetry and first-order autoregressive covariance structures was compared, and the results were found to be similar according to Akaike's criterion. Therefore, the values obtained with the compound-symmetry models are reported. Measurements of the microvascular density of bone marrow were grouped according to the length of treatment, and values were measured every 50 days for a total of seven times, including the pretreatment value. The natural logarithm of the values for the microvascular density of bone marrow was used in the analysis. Estimates for patients with no response and patients with a complete or nearly complete response (≥90 percent reduction in serum or urine paraprotein levels) were used to predict the response in terms of the microvascular density of bone marrow over time.

Improvements in important clinical measures were evaluated on the basis of the percent change from base line to the time of the maximal response or, for those without a response, the time at which treatment was discontinued. Spearman correlations were used to assess whether the changes within response groups were significant. For variables with no significant correlations, the signed-rank test was used to test the hypothesis within response groups that the change was significantly different from zero. All statistical tests were two-sided.

RESULTS

Decline in Paraprotein Levels

Timely escalations in the daily dose of thalidomide to 400 mg, 600 mg, and 800 mg were possible in 83 percent, 62 percent, and 47 percent of the patients, respectively; the proportions of patients who eventually reached these levels were 86 percent, 68 percent, and 55 percent, respectively (Table 1). In 27 patients (32 percent), the serum or urine paraprotein level declined by at least 25 percent, including 7 (8 percent) with a decline of at least 50 percent, 6 (7 percent) with a decline of at least 75 percent, and 6 (7 percent) with a decline of at least 90 percent; 2 patients had a complete remission (Table 2). The median interval between the start of treatment and a decrease in the paraprotein level of at least 25 percent was 29 days (range, 4 days to 6 months) (Fig. 1). Seventy-eight percent of the responses of this magnitude were apparent within two months; they were observed within four months in all but two patients with a response. More marked reductions in paraprotein, by at least 50 percent and 75 percent, occurred after a median of two and three months of therapy, respectively.

A low plasma-cell–labeling index (assessed as a continuous variable) was the only statistically significant variable associated with a response among both the group with at least a 25 percent decrease in paraprotein levels ($P=0.01$) and the group with at least a 50 percent decrease ($P=0.01$). Using the median plasma-cell–labeling index of 0.2 percent as a cutoff value, we found that 46 percent of patients with values below the median had a reduction in paraprotein levels of at least 25 percent, as compared with 9 percent of patients with higher values ($P<0.05$). On univariate analysis, deletion of chromosome 13 was predictive of an unfavorable response, but not on multivariate analysis.

Bone Marrow Response

Bone marrow samples were obtained after one to nine months of therapy (median, three) in 48 patients. A paraprotein response was associated with a bone marrow response in 81 percent of the patients who could be evaluated (Table 2). In seven of the eight patients with at least a 90 percent reduction in paraprotein levels, the concurrently examined bone marrow specimens contained less than 5 percent plasma cells. A decline in the percentage of plasma cells in bone marrow by at least 50 percent occurred in only 4 of 27 patients with no paraprotein response (15 percent) who had follow-up bone marrow examinations.

TABLE 2. PARAPROTEIN RESPONSE AND BONE MARROW RESPONSE.

PARAPROTEIN RESPONSE	NO. OF PATIENTS (% OF TOTAL)	ASSESSMENT OF BONE MARROW RESPONSE		CURRENT STATUS	
		TOTAL NO.*	NO. WITH RESPONSE (%)†	NO. WITH RELAPSE (%)	NO. WHO DIED (%)
Complete remission	2 (2)	2	2 (100)	0	0
≥90% decrease in paraprotein	6 (7)	6	5 (83)	2	2
≥75% decrease in paraprotein	6 (7)	5	3 (60)	3	1
≥50% decrease in paraprotein	7 (8)	4	4 (100)	3	0
≥25% decrease in paraprotein	6 (7)	4	3 (75)	4	3
Total	27 (32)	21	17 (81)	12 (44)	6 (22)
No response	57 (68)	27	4 (15)	—	30 (53)

*The response could not be evaluated in 6 of the patients with a paraprotein response and in 30 of the patients with no paraprotein response.

†A bone marrow response was defined as the presence of less than 5 percent plasma cells in bone marrow in patients who had a complete paraprotein response or at least a 90 percent reduction in paraprotein levels and as a reduction in plasma cells of at least 50 percent in patients with all other types of paraprotein responses.

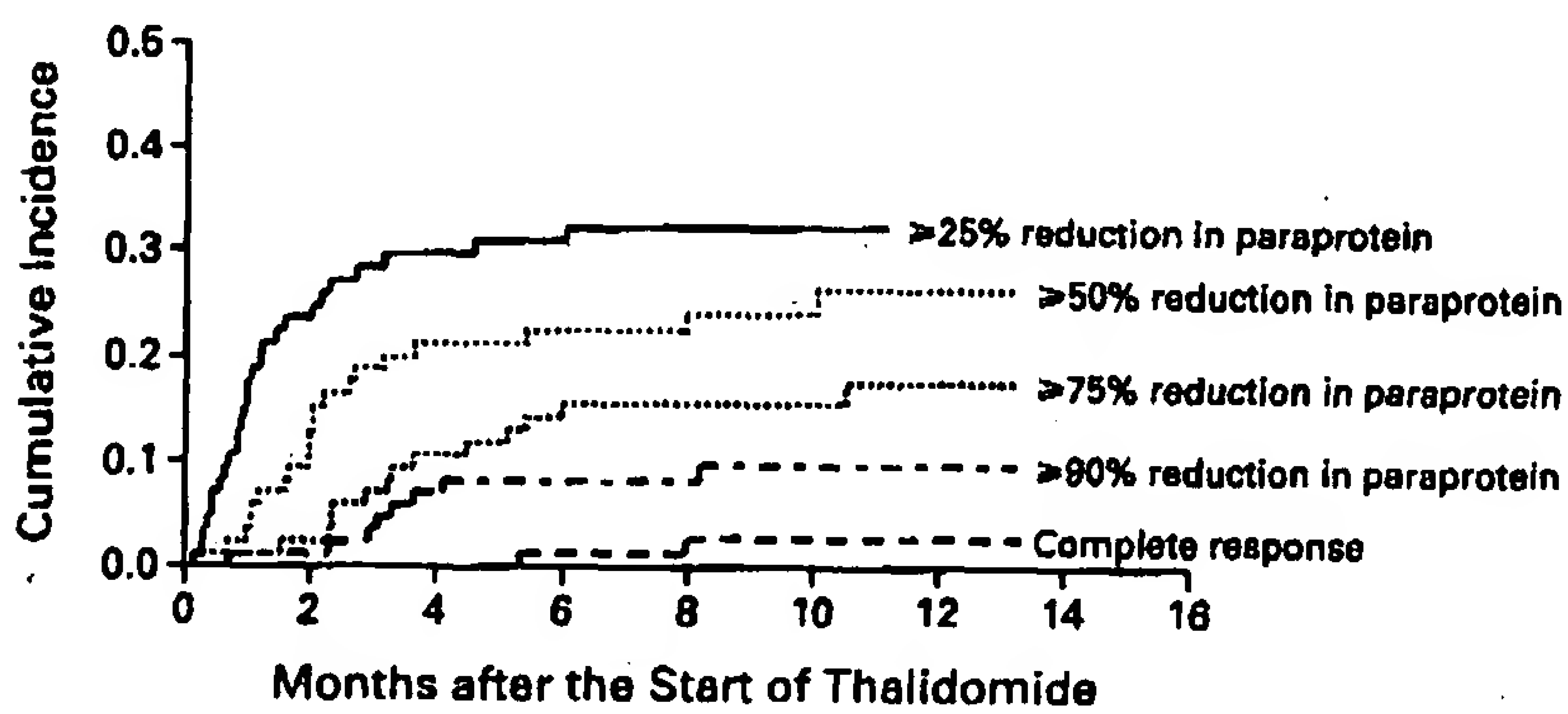


Figure 1. Times to Various Paraprotein Responses.

Among patients with a response, the median times to a reduction in the serum or urine paraprotein level of at least 25 percent, 50 percent, 75 percent, and 90 percent were one, two, four, and four months, respectively. Seventy-eight percent of the responses at the lowest level (≥25 percent reduction) were apparent within two months after the initiation of treatment.

Microvascular Density of Bone Marrow

The microvascular density of bone marrow was scheduled to be assessed every 50 days for a total of seven measurements, including the pretreatment value. At least one measurement of the microvascular density of bone marrow was made in 74 patients (88 percent); two or more measurements were made in 37 patients (44 percent). In all, measurements were made in 69 patients before treatment and (in 50-day increments) in 17 at time 2, in 22 at time 3, in 11 at time 4, in 12 at time 5, in 4 at time 6, and in 3 at time 7. The microvascular density of bone marrow and the percentage of plasma cells in bone marrow correlated significantly at all times except the last ($r>0.5$, $P\leq0.01$). Although the microvascular density of bone marrow decreased markedly in some patients with a complete or nearly complete remission, estimates of the slope were not significantly different from zero among those with a response ($P=0.39$) and those without a response ($P=0.22$).

Other Changes

The percent changes from base line to the time of the maximal response among patients with a response and the time of the last follow-up visit among those without a response were assessed for beta$_2$-microglobulin, C-reactive protein, lactic dehydrogenase, creatinine, albumin, and hemoglobin levels and the platelet count. Hemoglobin levels increased only in patients with a response (median increase, 11 percent; $P<0.001$ for the comparison with base-line values). Serum levels of beta$_2$-microglobulin rose (median in-

crease, 43 percent; P<0.001) and serum albumin levels fell (median decrease, 4 percent; P<0.001) significantly in patients with no response. Serum creatinine levels did not change significantly in patients with a response, and they increased by a median of 13 percent in those without a response (P<0.001).

Adverse Effects

Side effects reported by at least 10 percent of patients at most dose levels are listed in Table 3. Most adverse effects were mild or moderate (grade 1 or 2 according to the system of classification of the World Health Organization). Constipation, weakness or fatigue, and somnolence occurred in one third or more of the patients. Reports of grade 3 or 4 adverse effects were infrequent (less than 10 percent in all cases). One quarter of the patients had no appreciable side effects at the 200-mg dose, whereas virtually all patients had adverse effects of grade 1 or 2 at higher doses. Fewer than 5 percent of patients had grade 1 or 2 leukopenia at any dose, and grade 3 or 4 thrombocytopenia or anemia occurred in only three patients. In most of the patients who had no response, pretreatment anemia or thrombocytopenia did not worsen, whereas significant increases in the hemoglobin levels occurred in patients with a response. Nine patients could not tolerate thalidomide (four with a response and five with no response) and discontinued treatment after a median of 36 days (range, 10 to 241). In eight patients, an increase in serum creatinine levels of more than 50 percent was related to progressive disease, with increasing Bence Jones proteinuria. One of the patients with a response died suddenly on day 37 of treatment. The death was thought to be related to sepsis, although a possible contribution of thalidomide could not be ruled out.

Time to Progression, Event-free Survival, and Overall Survival

Of the 27 patients with a decrease in paraprotein levels of at least 25 percent, 12 had a recurrence of the disease. After a median follow-up of 14.5 months (range, 12 to 16), the median time to progression had not been reached. The disease in a mean (±SE) of 44±10 percent of patients was judged to have progressed at 12 months. The median event-free survival for all 84 patients was three months (Fig. 2). After 12 months of follow-up, 22±5 percent of the 84 patients remained event-free and 58±5 percent were alive. Nineteen patients were still receiving thalidomide 4 to 15 months after starting the treatment (median, 13), including 15 patients with a response and 4 with no response who had had some improvement in various disease indicators but who had not had a decrease in paraprotein levels of at least 25 percent. Multivariate analysis indicated that increases in lactic dehydrogenase levels (P=0.001), the plasma-cell–labeling index (P=0.006), and C-reactive protein levels (P=0.007) were all predictive of a brief period of event-free survival, whereas low albumin levels (P<0.001), the deletion of chromosome 13 (P=0.004), and high numbers of plasma cells in bone marrow (P=0.05) were associated with a relatively short overall survival.

Thalidomide was discontinued after a median of 52 days (range, 2 to 286) because of a lack of response in 53 patients (4 patients continued to receive the drug without a response) and because of relapse in 12 patients who had had a response. One patient who had a decrease in the paraprotein level of at least 25 percent and who had not previously received high-dose therapy subsequently underwent autologous stem-cell transplantation at his own request. As of June 17, 1999, 36 patients had died, including 30 patients without a response who died of progressive disease or complications of subsequent salvage therapy, as well as 6 patients with a response who subsequently relapsed and died of progressive disease (3) or toxicity from salvage therapy (3).

Table 3. Incidence of Grade 1 or 2 Adverse Effects.*

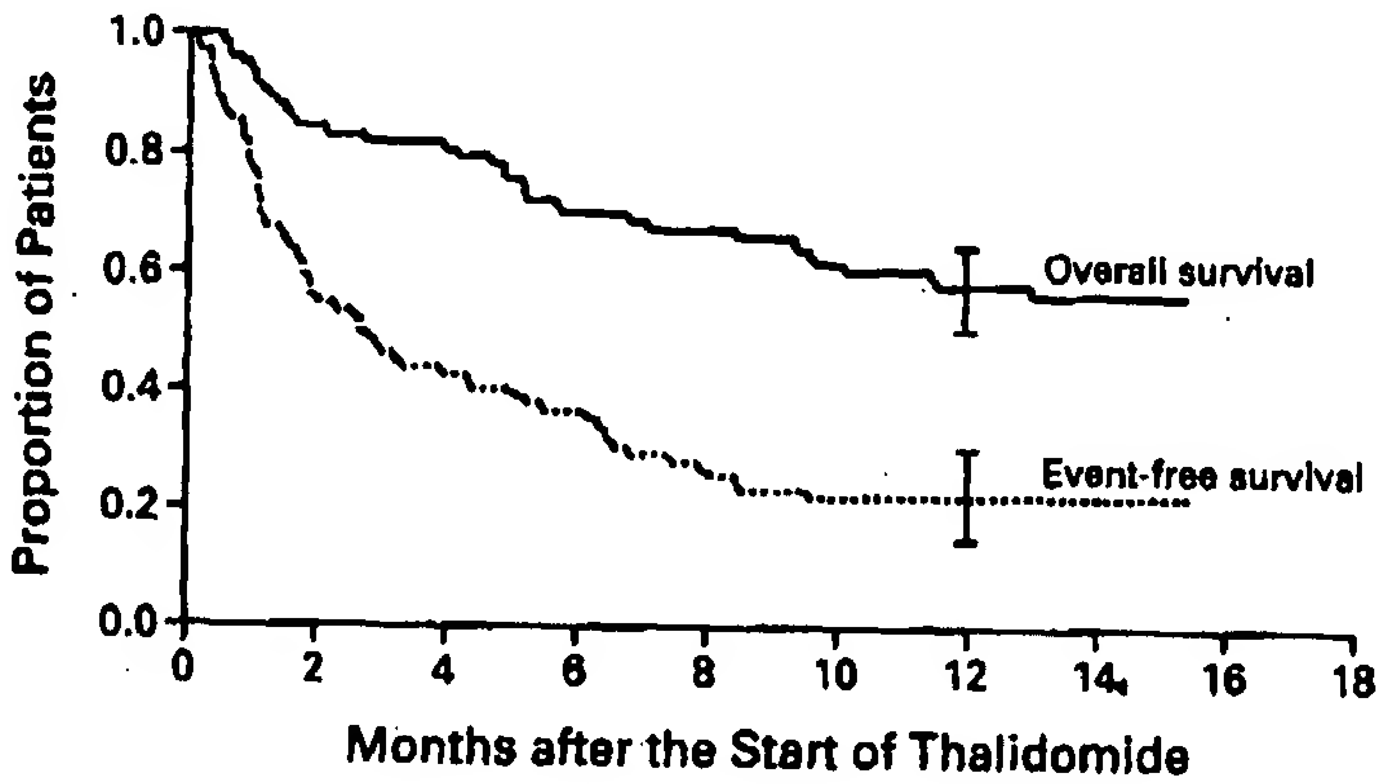
Adverse Effect	Dose of Thalidomide			
	200 mg/day (N=83)	400 mg/day (N=72)	600 mg/day (N=57)	800 mg/day (N=46)
	percentage of patients†			
Constipation	35	44	44	59
Weakness or fatigue	29	31	39	48
Somnolence	34	43	40	43
Tingling or numbness	12	14	19	28
Dizziness	17	25	23	28
Rash	16	18	21	26
Mood changes or depression	16	24	23	22
Incoordination	16	17	14	22
Tremors	10	13	19	22
Edema	6	10	12	22
Nausea	12	15	23	11
Headache	12	10	14	11

*The classification system of the World Health Organization was used. Grade 1 effects are mild, and grade 2 are moderate.

†Values are the percentages of patients at each dose level.

DISCUSSION

We found that thalidomide had substantial antitumor activity in patients with advanced myeloma. Ten percent of patients had complete or nearly complete remission, and 32 percent had a reduction in serum or urine paraprotein levels of at least 25 percent. In most patients, the decline in paraprotein levels was accompanied by a reduction in the percentage of plasma cells in bone marrow and an increase in hemoglobin levels, both of which are consistent with the



No. at Risk								
Overall survival	84	78	69	64	58	56	51	34
Event-free survival	84	65	39	32	24	19	18	11

Figure 2. Kaplan–Meier Estimates of Overall Survival and Event-free Survival.
Event-free survival was calculated from the start of thalidomide therapy to progression, removal from the study for any reason, death from any cause, or the last follow-up visit, whichever occurred first. I bars indicate standard errors at 12 months.

presence of a true antitumor effect. Although not examined quantitatively, bone pain decreased markedly in patients with a response. We did not evaluate lytic bone lesions, which seldom heal, even in patients with a sustained complete remission.

Thalidomide has a number of properties that could explain its activity in myeloma; it can alter the expression of adhesion molecules,[25] suppress the production of tumor necrosis factor α,[26] increase the production of interleukin-10,[27] and enhance cell-mediated immunity by directly stimulating cytotoxic T cells.[28] Its interactions with type 1 and type 2 helper T cells produce complex effects on the levels of cytokines such as interleukin-4, interleukin-5, and interferon-γ.[29] Thalidomide also increases the total number of lymphocytes as well as CD8+ and CD4+ T-cell counts, along with substantially increasing mean plasma levels of soluble interleukin-2 receptor.[29]

Thalidomide has been shown to inhibit angiogenesis induced by fibroblast growth factor and vascular endothelial growth factor in a rabbit-cornea micropocket assay[9] and a murine model of corneal vascularization.[10] It has also been shown to cause apoptosis of established tumor-associated angiogenesis in experimental models.[10] The bone marrow of patients with hematologic cancers shows extensive vascularity,[12,13] which has prognostic implications in myeloma.[14] The apparent lack of a consistent decrease in the microvascular density of bone marrow in patients in whom thalidomide had a marked antitumor effect requires further study. The persistence of extensive vascularization in some patients with a response is consistent with the finding of persistent neovascularity in patients with multiple myeloma who had a response to high-dose chemotherapy.[15] The production of angiogenic cytokines such as fibroblast growth factor and vascular endothelial growth factor by undetectable residual myeloma cells may sustain the increased microvascular density of bone marrow in patients considered to be in remission on the basis of bone marrow findings. The persistence of extensive vascularization in patients with a response makes it seem likely that the antimyeloma action of thalidomide depends on more than one of the actions of the drug outlined above. The mouse model of severe combined immunodeficiency, which can be used for the in vivo growth of primary human myeloma cells, is ideally suited to study the mechanisms by which thalidomide induces responses in myeloma.[30]

The antitumor properties of thalidomide are being evaluated in various malignant diseases,[31-35] although only limited efficacy data are available so far. Prolonged responses to thalidomide in some patients with advanced refractory disease suggest that the mechanism of action of thalidomide is distinctly different from that of the other agents active against myeloma. The absence of myelosuppressive and other important adverse effects suggests that thalidomide could be an ideal agent for use in combination with chemotherapy. Indeed, a complete remission has been achieved with such an approach in several patients with myeloma who had no response to treatment with either regimen alone.[36] This approach has also been shown to have greater antitumor activity than chemotherapy alone in a murine model of breast cancer.[37]

In our study, most patients had adverse effects, but the majority of these reactions were mild or moderate. Reducing the dose of thalidomide alleviated the effects in most cases, and only nine patients discontinued therapy altogether. The gradual reduction in

drowsiness and fatigue in some patients with continued treatment at the same dose (data not shown) suggests the occurrence of tachyphylaxis.

We conclude that thalidomide is active against multiple myeloma, even in patients who relapsed after repeated cycles of high-dose chemotherapy. Larger studies of thalidomide, its analogues, and other inhibitors of angiogenesis are therefore warranted in patients with myeloma and other cancers. We are currently evaluating thalidomide in combination with chemotherapy for patients with newly diagnosed multiple myeloma.

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Dr. Zeldis is an employee of Celgene Corporation, and Drs. Mehta and Singhal own stock in Celgene, which manufactures thalidomide.

We are indebted to Beth Wolmer for her persistence in recommending the clinical evaluation of thalidomide in the treatment of multiple myeloma; to the members of the myeloma data-management team for their dedication; and to Caran Swanson for her excellent secretarial assistance. This article is dedicated to the memory of Ira Wolmer, M.D.

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SHORT REPORT

Therapy with thalidomide in refractory multiple myeloma patients – the revival of an old drug

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Summary. We have treated 17 refractory or relapsed multiple myeloma patients resistant to chemotherapy with thalidomide at a dose of 200–800 mg/day. Eleven patients responded, five of whom had a very good partial response (> 75% decline in M protein) and another five exhibited a partial response (> 50% decline in M protein). Except for one patient, treatment was well tolerated with only mild side-effects. Thalidomide should be included in the therapeutic options for refractory myeloma.

Keywords: multiple myeloma, thalidomide.

Currently available therapies for multiple myeloma (MM) may produce temporary remissions but, ultimately, the disease has a fatal course. Patients with refractory or relapsed disease, in particular, have a very dismal prognosis, and even autologous or allogeneic stem cell transplants have either a limited impact on survival or are feasible only in selected patients (Anderson *et al*, 1999). Therefore, the recent communications from researchers at the University of Arkansas on the marked antimyeloma effect of thalidomide (Singhal *et al*, 1998) were most exciting and prompted us to conduct a pilot trial on a group of 17 patients with refractory or relapsed MM.

PATIENTS AND METHODS

Patients

The characteristics of patients are presented in Table I. There were 11 men and six women aged 44–68 (median 53) years. Thalidomide was started 1–11 (median 2·5) years after diagnosis. Patients had previously received one to six (median 3) regimens of chemotherapy, and nine had undergone high-dose chemotherapy with melphalan and etoposide with peripheral blood stem cell support. All patients had progressive disease not responding to chemotherapy. At least 1 month elapsed between the last therapy and the start of thalidomide treatment.

Treatment

Thalidomide was kindly supplied by Grünenthal (Thalidomid Grünenthal 100) and was given as recommended by Singhal *et al* (1998) starting at 200 mg daily and escalating according to tolerance every fortnight up to a maximum of 800 mg daily. Supportive care and monthly pamidronate infusions were the only other treatments allowed.

Evaluation of response

Only patients who received at least 6 weeks of treatment were included. All patients were assessed for their disease and questioned about the side-effects of thalidomide every 1–2 weeks. Complete blood counts, serum and M protein levels and urinary Bence Jones levels were performed regularly. Response was assessed according to the maximal decline in the M protein in serum or urine. Complete remission (CR) was defined as the disappearance of the M protein and normalization of all laboratory values; very good partial response (VGPR) was defined as ≥ 75% decline in the M protein; partial response (PR) was defined as ≥ 50% decline in the M protein; minimal response (MR) was defined as > 25% decline in the M protein. Non-responders had either stable (SD) or progressive disease (PD). Performance status was assessed according to the Zubrod scale and pain severity by the WHO three-step pain ladder.

RESULTS

Table II summarizes the maximal thalidomide dosage administered, the duration of treatment and the response to treatment. Eleven out of the 17 patients (64%) responded: five had VGPR; five had PR and one had MR, while six failed to respond (five with PD and one with SD), one of whom died from progressive disease (case 2). One PR patient died from

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Table I. Patient characteristics.

No.	Gender	Age (years)	M protein	Time from diagnosis (years)	Previous treatment
1	F	46	IgG-κ	7	VAD, HD-CTX, SCT
2	M	48	BJ-κ	3	VMCP/VBAP, VAD, CTX, SCT
3	M	44	IgA-λ	4·5	VAD, SCT, DCEP, HD-CTX
4	F	52	IgG-κ	4	VAD, SCT, VAD
5	M	58	Non-secreting	3·5	VAD, SCT
6	F	49	IgG-κ	3·5	VMCP/VBAP, VAD, SCT, MP, IDA,CTX
7	F	49	IgG-κ	2	VAD, DEX, HD-CTX, MP
8	F	53	IgG-κ	1·5	VAD, HD-CTX, CM
9	M	66	IgG-κ	2	VAD, MP, CTX
10	M	64	IgG-κ	2	VAD, HD-CTX, SCT
11	M	47	BJ-κ	2	VAD, SCT, VAD, HD-CTX, DCEP
12	M	54	IgG-κ	2·5	VAD, CTX, SCT, DCEP
13	M	44	IgG-λ	2	VAD, HD-CTX, HD-M
14	F	68	IgA	4	MP
15	M	61	BJ-λ	1	VAD, HD-CTX, MP
16	M	63	IgG-κ	11	VMCP, MP, CTX, DEX, IDA
17	M	67	IgA-λ	2·5	VMCP, DEX, HD-CTX, DCEP, CM

VAD, vincristine, doxorubicin, dexamethasone; HD-CTX, high-dose cyclophosphamide; SCT, high-dose chemotherapy with stem cell transplantation; VMCP/VBAP, vincristine, melphalan, cyclophosphamide, prednisone/vincristine, lomustine, doxorubicin, prednisone; MP, melphalan, prednisone; CTX, cyclophosphamide; DCEP, dexamethasone, cyclophosphamide, etoposide, cisplatin; IDA, idarubicin; DEX, dexamethasone; CM, cyclophosphamide, melphalan with peripheral blood stem cell support.

sepsis and renal failure (case 1). In all responders, except for the patient with renal failure (case 1) and the patient who discontinued treatment because of toxicity (case 14), there was a concomitant rise in the haemoglobin to normal levels.

The earliest time interval between the start of treatment and a measurable decrease in M protein was 2–3 weeks. All responders had a clear decrease in the M protein after 1 month of treatment. Among the 11 responders, the

Table II. Thalidomide treatment characteristics and response to treatment.

No.	Maximal dose (mg/day)	Treatment duration (months)	Baseline and maximal response M protein (g/l) Before	After	Haemoglobin (g/dl) Before	After	Response*	Comments
1	600	3	83	26	6·8	8·6	PR	Died from renal failure, sepsis
2	800	2	1·0†	7·0†	8·9	9·3	PD	Died from progressive disease
3	800	6	52	57	8·4	8·4	PD	
4	600	5	40	09	9·6	13·6	VGPR	Normal BM
5	800	3	35‡	<5‡	10·6	13·7	VGPR	Normal BM
6	400	6	45	9·0	9·0	12·6	VGPR	
7	800§	7	48	2·0	6·9	12·3	VGPR	
8	800	2	41	53	9·3	9·3	PD	
9	800	5	68	45	8·0	12·0	PR	
10	800§	4	25	11	9·8	12·4	PR	
11	600	3	7·2†	4·7†	9·6	11·0	MR	
12	600	3	42	10	9·7	12·1	VGPR	Normal BM
13	600	3	43	22	10·7	12·2	PR	Response duration 2 months
14	600§	2·5	32	19	7·0	8·5	PR	Discontinued because of toxicity
15	800§	4	8·0†	8·3†	9·9	9·2	PD	Renal failure
16	200	3	85	109	8·2	8·9	PD	
17	600	2	36	36	8·7	9·0	SD	

*See text for definition of response.
†BJ proteinuria (g/day).
‡Non-secreting myeloma (percentage of BM plasma cells).
§Dose reduction required eventually.

performance status was unchanged in five and improved from scale 1 (symptomatic, fully ambulatory) to 0 (asymptomatic) in four patients and from scale 2 (symptomatic, in bed < 50% of the day) to 0 in one patient. The pain score was unchanged in six responders, all of whom were free of pain before and during the study, and decreased in four patients from scale 1 (requiring non-opioid analgesics) to pain-free status. One patient who was on opioid analgesics (scale 2) became pain free. All patients were normocalcaemic before and during treatment.

Treatment was generally well tolerated, but one PR patient (case 14) had significant neurological signs and symptoms requiring discontinuation of the drug. Two months later, her disease progressed, and thalidomide was restarted again with response. Milder side-effects were seen in most patients: 11 complained of somnolence; five of severe tiredness; and five had constipation. One patient had spells of dizziness, and one had bradycardia with fluctuations in blood pressure attributed to autonomic neuropathy. In four patients, side-effects were severe enough to require dose reduction, but response was maintained in the two responders despite dose reduction. In one failing patient, the dosage could not be increased beyond 200 mg daily. Of the 11 responders, nine have continued their response for 3–8 months. In one (case 13), the response lasted for 2 months only, indicating the acquisition of resistance.

DISCUSSION

Most malignant tumours depend on angiogenesis to proliferate and metastasize (Folkman, 1995). Angiogenesis plays an important role in haematological malignancies as well. Several studies have found that bone marrow angiogenesis was observed in patients with active MM and correlated with proliferating activity and prognosis (Vacca *et al*, 1994, 1999; Munshi *et al*, 1998). Thalidomide has an inhibitory effect on angiogenesis, as shown in an animal model (D'Amato *et al*, 1994), and this could be the mechanism of its beneficial action in MM patients. However, thalidomide also has many other effects, including immunomodulation, which have been used in treating chronic graft versus host disease (GVHD) and erythaema nodosum leprosum, and these could play a role in the response of myeloma patients.

Our small pilot study confirmed the initial data presented by Singhal *et al* (1998) and showed a significant response in refractory MM patients. The effect of thalidomide in some of our patients was quite dramatic and changed them from symptomatic and transfusion dependent to being able to live an almost normal life. Thalidomide should therefore be included in the therapeutic options in refractory MM and should be explored in controlled studies in other phases of the disease as a single agent or in combination with chemotherapy.

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We are most grateful to Dr K. Zwingenberger from Grünenthal, Aachen, Germany, for providing thalidomide for our patients.

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Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy

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Although thalidomide (Thal) was initially used to treat multiple myeloma (MM) because of its known antiangiogenic effects, the mechanism of its anti-MM activity is unclear. These studies demonstrate clinical activity of Thal against MM that is refractory to conventional therapy and delineate mechanisms of anti-tumor activity of Thal and its potent analogs (immunomodulatory drugs [IMiDs]). Importantly, these agents act directly, by inducing apoptosis or G1 growth arrest, in MM cell lines and in patient MM cells that are resistant to melphalan, doxorubicin, and dexamethasone (Dex). Moreover, Thal and the IMiDs enhance the anti-MM activity of Dex and, conversely, are inhibited by interleukin 6. As for Dex, apoptotic signaling triggered by Thal and the IMiDs is associated with activation of related adhesion focal tyrosine kinase. These studies establish the framework for the development and testing of Thal and the IMiDs in a new treatment paradigm to target both the tumor cell and the microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease. (Blood. 2000;96:2943-2950)

Introduction

Thalidomide (Thal) was originally used in Europe for the treatment of morning sickness in the 1950s but was withdrawn from the market in the 1960s because of reports of teratogenicity and phocomelia associated with its use. The renewed interest in Thal stems from its broad spectrum of pharmacologic and immunologic effects.[1] Because of its immunomodulatory and antiangiogenic effects, it has been used to effectively treat erythema nodosum leprosum, an inflammatory manifestation of leprosy.[2] Potential therapeutic applications span a wide spectrum of diseases, including cancer and related conditions, infectious diseases, autoimmune diseases, dermatologic diseases, and other disorders such as sarcoidosis, macular degeneration, and diabetic retinopathy.[3] Recent reports of increased bone marrow (BM) angiogenesis in multiple myeloma (MM),[4,5] coupled with the known antiangiogenic properties of Thal,[6] provided the rationale for its use to treat MM.[7] Importantly, Thal induced clinical responses in 32% of MM patients whose disease was refractory to conventional and high-dose therapy,[7] suggesting that it can overcome drug resistance because of its alternative mechanisms of anti-MM activity. Besides alkylating agents and corticosteroids, Thal now, therefore, represents the third distinct class of agents useful in the treatment of MM.

Given its broad spectrum of activities, Thal may be acting against MM in several ways.[8] First, Thal may have a direct effect on the MM cell and/or BM stromal cell to inhibit their growth and survival. For example, free radical–mediated oxidative DNA damage may play a role in the teratogenicity of Thal[9] and may also have anti-tumor effects. Second, adhesion of MM cells to BM stromal cells both triggers secretion of cytokines that augment MM cell growth and survival[10-12] and confers drug resistance[13]; Thal modulates adhesive interactions[14] and, thereby, may alter tumor cell growth, survival, and drug resistance. Third, cytokines secreted into the BM microenvironment by MM and/or BM stromal cells, such as interleukin (IL)-6, IL-1β, IL-10, and tumor necrosis factor (TNF)–α, may augment MM cell growth and survival,[12] and Thal may alter their secretion and bioactivity.[15] Fourth, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor 2 (bFGF-2) are secreted by MM and/or BM stromal cells and may play a role both in tumor cell growth and survival, as well as BM angiogenesis.[5,16] Given its known antiangiogenic activity,[6] Thal may inhibit activity of VEGF, bFGF-2, and/or angiogenesis in MM. However, Singhal et al.[7] observed no correlation of BM angiogenesis with response to Thal, suggesting that it may not be mediating anti-MM activity by its antiangiogenic effects. Finally, Thal may be acting against MM by its immunomodulatory effects, such as induction of a Th1 T-cell response with secretion of interferon gamma (IFN-γ) and IL-2.[17] Already 2 classes of Thal analogs have been reported, including phosphodiesterase 4 inhibitors that inhibit TNF-α but do not enhance T-cell activation (selected cytokine inhibitory drugs [SelCIDs]) and others that are not phosphodiesterase 4 inhibitors but markedly stimulate T-cell proliferation as well as IL-2 and IFN-γ production (immunomodulatory drugs [IMiDs]).[15]

In this study, we have begun to characterize the mechanisms of activity of Thal and these analogs against human MM cells. Delineation of their mechanisms of action, as well as mechanisms of resistance to these agents, will both enhance understanding of MM disease pathogenesis and derive novel treatment strategies.

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Materials and methods

MM-derived cell lines and patient cells

Dexamethasone (Dex)-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). Doxorubicin (Dox)-, mitoxantrone (Mit)-, and melphalan (Mel)-sensitive and -resistant RPMI-8226 human MM cells were kindly provided by Dr William Dalton (Moffitt Cancer Center, Tampa, FL). RPMI-8226 cells resistant to Dox, Mit, and Mel included Dox 6 and Dox 40 cells, MR20 cells, and LR5 cells, respectively. Hs Sultan human MM cells were obtained from the American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI-1640 media (Sigma Chemical, St Louis, MO) that contained 10% fetal bovine serum, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). Drug-resistant cell lines were cultured with either Dox, Mit, Mel, or Dex to confirm their lack of drug sensitivity. MM patient cells (96% $CD38^+CD45RA^-$) were purified from patient BM samples, as previously described.[18]

Thal and analogs

Thal and analogs (Celgene, Warren, NJ) were dissolved in DMSO (Sigma) and stored at −20°C until use. Drugs were diluted in culture medium (0.0001 to 100 μM) with < 0.1% DMSO immediately before use. The Thal analogs used in this study were 4 SelCIDs (SelCIDs 1, 2, 3, and 4), which are phosphodiesterase 4 inhibitors that inhibit TNF-α production and increase IL-10 production from lipopolysaccharide (LPS)–stimulated peripheral blood mononuclear cells (PBMCs) but do not stimulate T-cell proliferation; and 3 IMiDs (IMiD1, IMiD2, and IMiD3), which do stimulate T-cell proliferation, as well as IL-2 and IFN-γ secretion, but are not phosphodiesterase 4 inhibitors. The IMiDs also inhibit TNF-α, IL-1β, and IL-6 and greatly increase IL-10 production by LPS-stimulated PBMCs.[15]

DNA synthesis

DNA synthesis was measured as previously described.[19] MM cells (3×10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, Thal, SelCID1, SelCID2, SelCID3, SelCID4, IMiD1, IMiD2, IMiD3, and/or recombinant IL-6 (50 ng/mL) (Genetics Institute, Cambridge, MA) for 48 hours at 37°C. DNA synthesis was measured by [^{3}H]-thymidine (^{3}H-TdR; NEN Products, Boston, MA) uptake. Cells were pulsed with ^{3}H-TdR (0.5 μCi/well) during the last 8 hours of 48-hour cultures, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted by using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). All experiments were performed in triplicate.

Colorimetric assays were also performed to assay drug activity. Cells from 48-hour cultures were pulsed with 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Chemicon International Inc, Temecula, CA) to each well for 4 hours, followed by 100 μL isopropanol that contained 0.04 HCl. Absorbance readings at a wavelength of 570 nm were taken on a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Cell cycle analysis

MM cells (1×10^6) cultured for 72 hours in media alone, Thal, IMiD1, IMiD2, and IMiD3 were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, and pretreated with 10 μg/mL of RNAse (Sigma). Cells were stained with propidium iodide (PI; 5 μg/mL; Sigma), and cell cycle profile was determined by using the program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies.[20]

Detection of apoptosis

In addition to identifying sub-G1 cells as described above, apoptosis was also confirmed by using annexin V staining. MM cells were cultured in media (0.01% DMSO) or with 10 μmol/L of Thal or 1 μmol/L IMiD1, IMiD2, and IMiD3 at 37°C for 72 hours, with addition of drugs at 24-hour intervals. Cells were then washed twice with ice-cold PBS and resuspended (1×10^6 cells/mL) in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). MM cells (1×10^5) were incubated with annexin V-FITC (5 μL; Pharmingen, San Diego, CA) and PI (5 μg/mL) for 15 minutes at room temperature. Annexin V+PI− apoptotic cells were enumerated by using the Epics cell sorter (Coulter).

Immunoblotting

MM cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed using lysis buffer: 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton-X 100, 30 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 5 μg/mL leupeptin, and 5 μg/mL aprotinin. For detection of p21, cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-p21 antibody (Ab; Santa Cruz Biotech, Santa Cruz, CA). The membrane was stripped and reprobed with anti–alpha tubulin Ab (Sigma) to ensure equivalent protein loading. For detection of p53, cell lysates were prepared from MM cells (2×10^7) with the use of lysis buffer. Lysates were incubated with anti-mutant (mt) or wild-type (wt) p53 monoclonal Abs (Calbiochem, San Diego, CA) and then immunoprecipitated overnight with protein A Sepharose (Sepharose CL-4B; Pharmacia, Uppsala, Sweden). Immune complexes were analyzed by immunoblotting with horseradish peroxidase–conjugated anti-p53 Ab reactive with both mt and wt p53 (Calbiochem).

To characterize growth signaling, immunoblotting was also done with anti-phospho–specific MAPK Ab (New England Biolabs, Beverly, MA) in the presence or absence of IL-6 (Genetics Institute) and/or the MEK 1 inhibitor PD98059 (New England Biolabs), as in prior studies.[21] Antigen-antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Blots were stripped and reprobed with anti-ERK2 Ab (Santa Cruz Biotech) to ensure equivalent protein loading.

To characterize apoptotic signaling, MM cells were cultured with 100 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed in 1 mL of lysis buffer (50 mmol/L Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin), as in prior studies.[22] Lysates were incubated with anti-related adhesion focal tyrosine kinase (RAFTK) Ab for 1 hour at 4°C and then for 45 minutes after the addition of protein G–agarose (Santa Cruz Biotech). Immune complexes were analyzed by immunoblotting with anti-P-Tyr Ab (RC20; Transduction Laboratories, Lexington, KY) or anti-RAFTK Abs. Proteins were separated by electrophoresis in 7.5% SDS-PAGE gels, transferred to nitrocellulose paper, and analyzed by immunoblotting. The antigen-antibody complexes were visualized by chemiluminescence.

Statistical analyses

Statistical significance of differences observed in drug-treated versus control cultures was determined by using the Student t test. The minimal level of significance was $P < .05$.

Results

Treatment of MM patients with Thal

Seventeen (39%) of 44 patients with MM treated at our institute responded to Thal (Table 1). This response included 6 men and 11 women. These patients had received a median of 4 (1-9) prior treatment regimens, and 10 patients had a prior high-dose therapy and hematopoietic stem cell transplant. One patient achieved

Table 1. Response to thalidomide in multiple myeloma*

Patient	Sex†	Prior therapies	Prior stem cell transplant	Maximum change M protein‡	Duration of thalidomide therapy (mo)	Maximum daily dose thalidomide	Current status (daily thalidomide dose)
1	M	3	Yes	− 58%(PR)	8.5	200 mg	Continued response (200 mg)
2	F	5	No	− 78%(PR)	6.0	400 mg	Continued response (400 mg)
3	F	1	Yes	+ 16%(SD)	6.5	100 mg	Continued response (100 mg)
4	M	6	No	− 56%(PR)	9.0	200 mg	Continued response (200 mg)
5	F	1	No	− 62%(PR)	5.5	200 mg	Continued response (50 mg)
6	F	5	Yes	− 100%(CR)	13	500 mg	Continued response (50 mg)
7	M	9	Yes	− 54%(PR)	10	800 mg	Progressed (800 mg)
8	F	5	Yes	− 68%(PR)	4.0	200 mg	Continued response, discontinued
9	F	5	No	− 90%(PR)	7.5	400 mg	Continued response (400 mg)
10	M	5	Yes	− 9%(SD)	1.5	400 mg	Progressed
11§	F	4	Yes	− 59%(PR)	5.5	400 mg	Progressed
12§	M	4	Yes	− 64%(PR)	7.0	400 mg	Progressed
13§	F	3	Yes	− 14%(SD)	4.5	400 mg	Progressed
14§	F	2	Yes	− 55%(PR)	4.0	800 mg	Continued response (800 mg)
15	F	1	No	− 31%(SD)	6.0	400 mg	Continued response (400 mg)
16	F	1	No	− 12%(SD)	4.5	400 mg	Progressed
17	M	2	No	− 55%(PR)	6.0	200 mg	Continued response (100 mg)

*As of January 1, 2000.

†Male (M) or female (F).

‡Partial response (PR) is ≥ 50% decrease in M protein; complete response (CR) is absence of M protein on immunofixation and normal bone marrow biopsy; stable disease (SD) is ≤ 50% decrease in M protein; progression is ≥ 25% increase in M protein or progressive clinical disease.

§Also received decadron therapy.

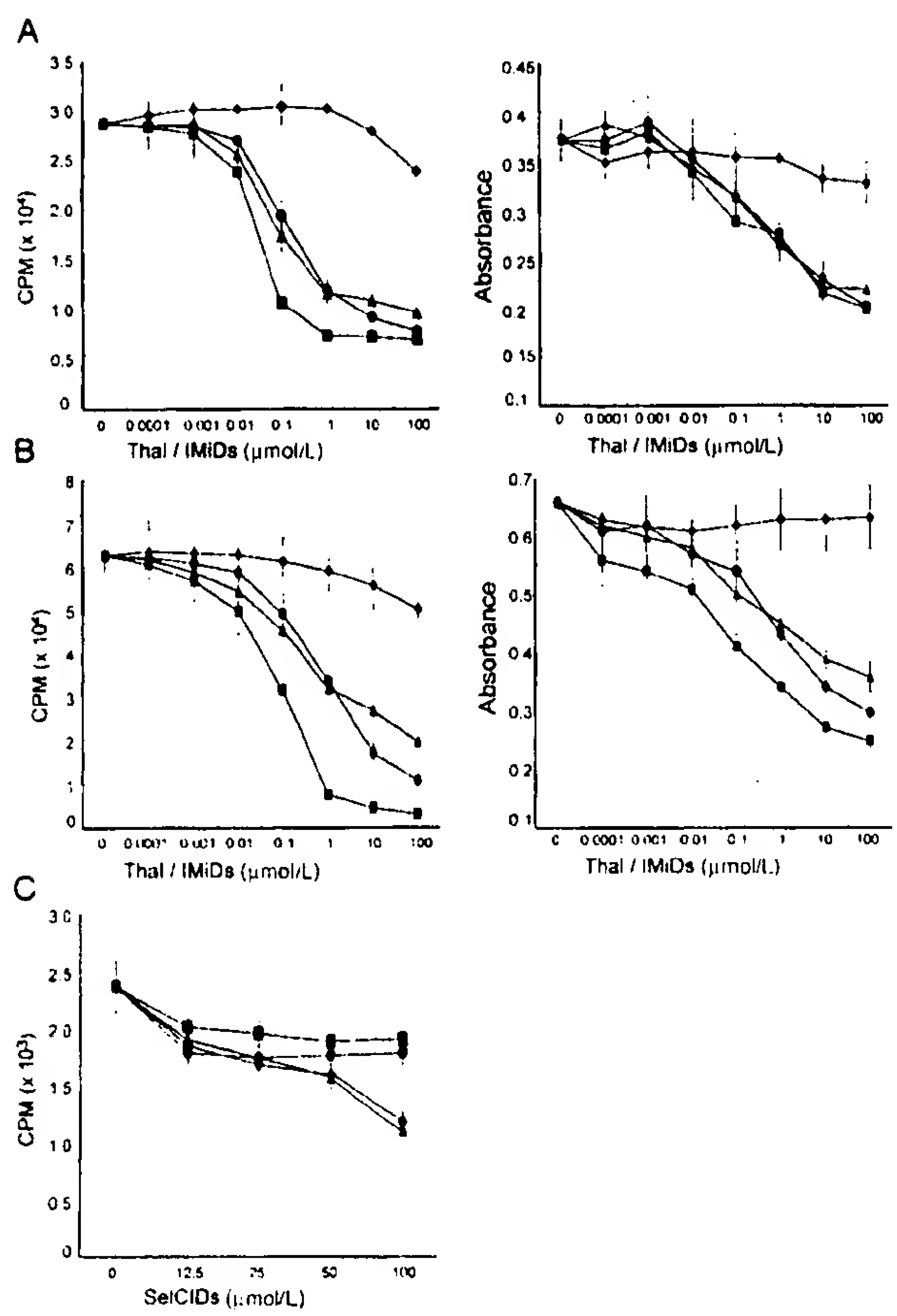

Figure 1. Effect of Thal and analogs on DNA synthesis of MM cell lines and patient cells. MM.1S (A) and Hs Sultan (B) cells were cultured with increasing concentrations (0.0001-100 μM) of Thal (◆), IMiD1 (■), IMiD2 (●), and IMiD3 (▲). (C) MM.1S cells were cultured with increasing concentrations (12.5-100 μM) of SelCID1 (◆), SelCID2 (■), SelCID3 (▲), and SelCID4 (●). In each case ^{3}H-TdR uptake (left panels) or MTT cleavage (right panels) were measured during the last 8 and 4 hours, respectively, of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) or absorbance of triplicate cultures.

complete response (absence of monoclonal protein on immunofixation and normal BM biopsy), 11 patients achieved partial response (> 50% decrease in monoclonal protein), and 5 patients achieved stable disease (< 50% decrease in monoclonal protein). Patients received a median of 400 mg (range, 100-800 mg) maximum dose of daily Thal for a median of 6 months (range, 1.5-13 months). As of January 1, 2000, 11 patients have continued response at a median of 6 months (range, 4-13 months), and 6 patients have progressed at a median of 4.5 months (range, 1.5-10 months).

Effect of Thal and analogs on DNA synthesis by MM cell lines and patient MM cells

The effect of Thal and its analogs, including IMiD1, IMiD2, IMiD3, SelCID1, SelCID2, SelCID3, and SelCID4, on DNA synthesis of MM cell lines (MM.1S, Hs Sultan, U266, and RPMI-8226) was determined by measuring ^{3}H-TdR uptake during the last 8 hours of 48-hour cultures, in the presence or absence of drug at various concentrations. IMiD1, IMiD2, and IMiD3 inhibited ^{3}H-TdR uptake of MM.1S (Figure 1A) and Hs Sultan (Figure 1B) cells in a dose-dependent fashion. Fifty percent inhibition of proliferation of MM.1S cells was noted at 0.01-0.1 μmol/L IMiD1, 0.1-1.0 μmol/L IMiD2, and 0.1-1.0 μmol/L IMiD3 ($P < .001$). Fifty percent inhibition of proliferation of Hs Sultan cells was noted at 0.1 μmol/L IMiD1, 1.0 μmol/L IMiD2, and 1.0 μmol/L IMiD3 ($P < .001$). In contrast, only 15% and 20% inhibition in MM.1S and Sultan cells, respectively, were observed in cultures at even higher concentrations (100 μmol/L) of Thal. No significant inhibition of DNA synthesis of U266 MM cells was noted in cultures with 0.001 to 100 μmol/L Thal or these IMiDs (data not shown). The effects of these drugs on proliferation were confirmed by using MTT assays for MM.1S cells (Figure 1A) and Hs Sultan cells (Figure 1B). Although there was also a dose-dependent inhibition of proliferation of MM.1S cells by SelCIDs, 50% inhibition was observed only at high doses (100 μmol/L) for only 2 of the 4 SelCIDs (SelCIDs 1 and 3, Figure 1C). Further studies, therefore, focused on Thal and the IMiDs.

Effect of Thal and analogs in DNA synthesis of MM cells resistant to conventional therapy

To examine whether there was cross-resistance between Thal and the IMiDs with conventional therapies, RPMI-8226 MM cells resistant to Dox (Dox6 and Dox40 cells), Mit (MR20 cells), or Mel (LR5 cells), and MM.1R cells resistant to Dex were similarly studied. Proliferation of Dox6 and Dox40, MR20, LR5, or MM1.R cells is unaffected by culture with 60 nmol/L and 400 nmol/L Dox, 20 nmol/L Mit, 5 μmol/L Mel, and 1 μmol/L Dex, respectively (data not shown). Importantly, ^{3}H-TdR uptake of Dox6, Dox40, MR20, or LR5 was inhibited in cultures with Thal and the IMiDs in a dose-dependent manner (1-100 μmol/L) versus media alone cultures (Figure 2A-D). For example, 10 μmol/L IMiD1 blocked proliferation of Dox6, Dox40, MR20, and LR5 cells by 20%, 33%, 32%, and 21%, respectively ($P < .001$). The IMiDs similarly inhibited DNA synthesis of MM.1R cells in a dose-dependent fashion, with more than 50% inhibition at more than 1 μmol/L IMiD1 ($P < .001$; Figure 2E). These data suggest independent mechanisms of resistance to Dox, Mit, Mel, and Dex versus Thal and its analogs.

Effect of Dex and IL-6 on response of MM cells to Thal and the ImiDs

To determine whether the effects of Thal and the IMiDs are additive with conventional therapies, we next examined the effect of Dex (0.001-0.1 μmol/L) together with 1 μmol/L Thal or IMiDs on proliferation of Dex-sensitive MM.1S cells. As can be seen in Figure 3A, the IMiDs (1 μmol/L) significantly inhibited ^{3}H-TdR uptake of MM.1S cells (60%-75% block, $P < .01$), and Dex (0.001-0.1 μmol/L) increased this inhibition in a dose-dependent fashion. For example, doses of 0.001 to 0.01 μmol/L Dex added to 1 μmol/L IMiD1 increased the inhibition of proliferation by 35% relative to cultures with 1 μmol/L IMiD1 alone ($P < .01$). Given the additive effects of Dex and the IMiDs, as well as the known role of IL-6 as a growth factor and specific inhibitor of Dex-induced MM cell apoptosis,[19,22,23] we also examined whether exogenous IL-6 could overcome the inhibition of DNA synthesis triggered by Thal and the IMiDs. Figure 3B demonstrates that IL-6 (50 ng/mL) triggers DNA synthesis of MM.1S cells in cultures with media alone, as well as in cultures with the IMiDs (0.1 and 1 μmol/L).

Effect of Thal and analogs on DNA synthesis of patient MM cells

The effect of Thal and the IMiDs on DNA synthesis of patient MM cells was next examined (Figure 4). As was true for MM.1S and Hs Sultan MM cell lines, ^{3}H-TdR uptake of patients' MM cells was also inhibited by IMiDs (0.1-100 μmol/L) in a dose-dependent fashion, whereas the inhibitory effect of Thal, even at 100 μmol/L, was not significant. Fifty percent inhibition of MM patient cells was observed at 100 μmol/L (Figure 4A) and 1 μmol/L (Figure 4B) IMiD1, respectively ($P < .001$).

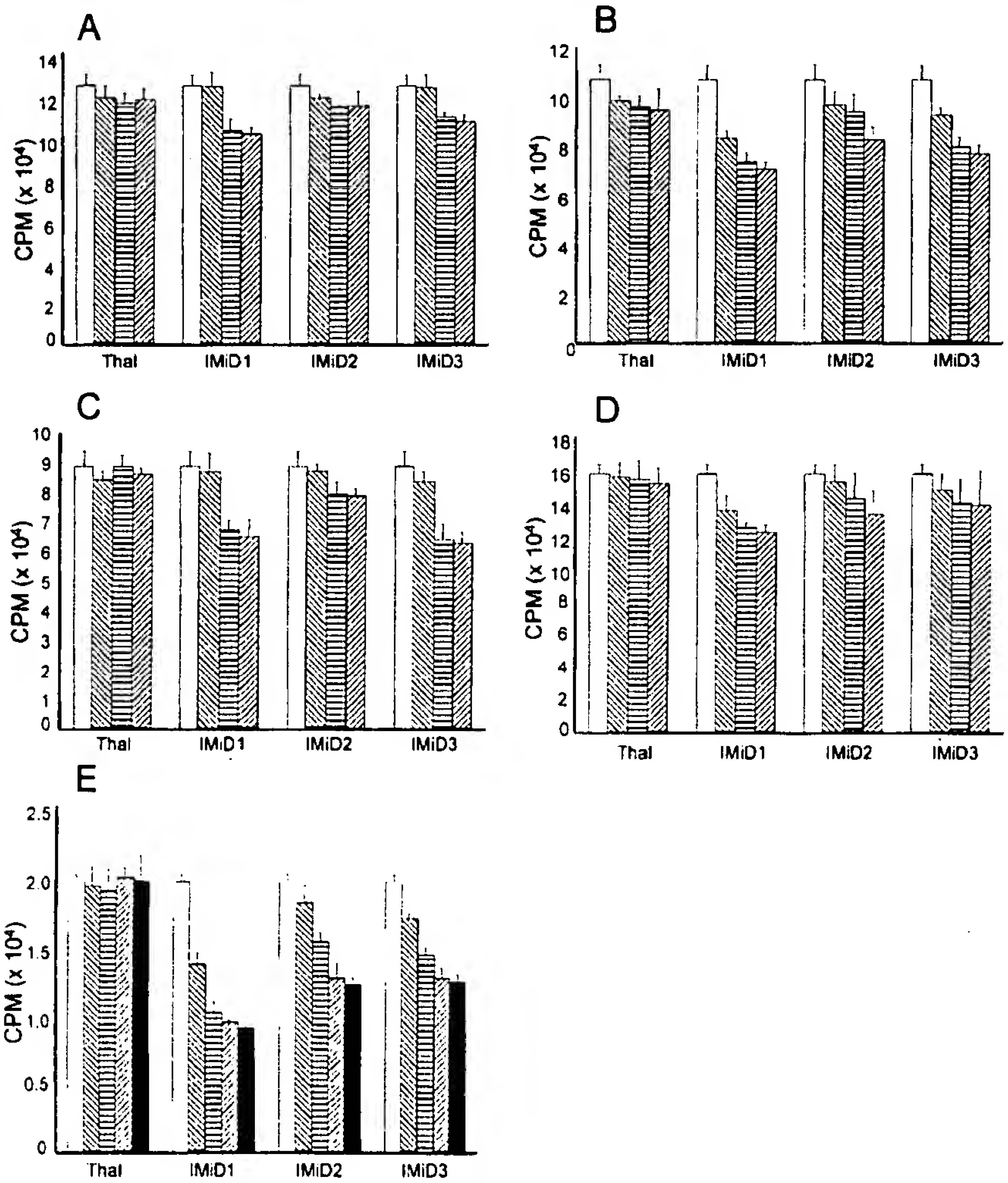

Figure 2. Effect of Thal and analogs on DNA synthesis of MM cells resistant to conventional therapy. Dox-resistant Dox6 (A) and Dox 40 (B), Mit-resistant (MR20; C), and Mel-resistant (LR5; D) cells were cultured with control media (□) or 1 μmol/L (▨), 10 μmol/L (▤), 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures. (E) Dex-resistant MM.1R cells were cultured in control media (□) or with 0.1 μmol/L (▨), 1 μmol/L (▤), 10 μmol/L (▧), or 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3.

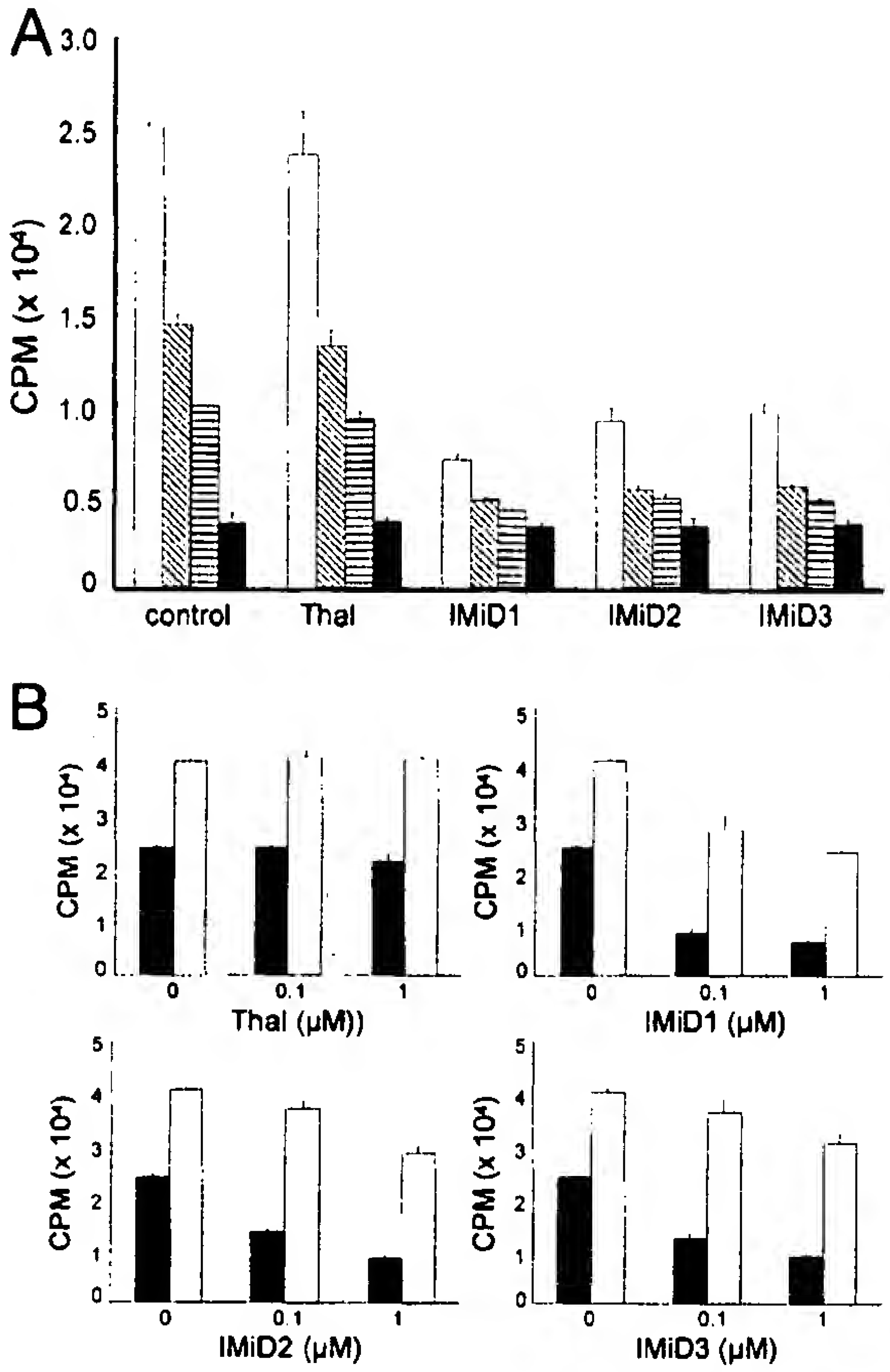

Figure 3. Effect of Dex and IL-6 on response of MM cells to Thal and the IMiDs. (A) MM.1S cells were cultured with 1.0 μM Thal, IMiD1, IMiD2, or IMiD3 in control media alone (□) or with 0.001 (▨), 0.01 (▤), and 0.1 μmol/L (■) Dex. (B) MM.1S cells were cultured in control media alone and with 0.1 and 1.0 μmol/L Thal, IMiD1, IMiD2, or IMiD3 either in the presence (□) or absence (■) of IL-6 (50 ng/mL). In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells

To further analyze the mechanism of Thal- and IMiD-induced inhibition of DNA synthesis and to determine whether these drugs induced apoptosis of MM cells, we first examined the cell cycle profile of MM.1S, Hs Sultan cells, and patient MM cells cultured with media alone, Thal (10 μmol/L), or the IMiDs (1 μmol/L). Cells were harvested from 72-hour cultures and stained with PI. As shown in Figure 5A, all 3 IMiDs, and Thal to a lesser extent, increased sub-G1 MM.1S cells. Induction of apoptosis occurred at the dose-response curve noted for inhibition of proliferation. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for triggering increased sub-G1 cells. In contrast, no increase in sub-G1 cells was observed in cultures of Hs Sultan cells or of patient MM cells with Thal or the IMiDs. Importantly, Thal and the IMiDs induced G1 growth arrest in both Hs Sultan cells and in AS patient MM cells.

To confirm these results, we performed annexin V staining of cells in these cultures. As can be seen in Figure 5B, the percentage of annexin V–positive cells in cultures of MM.1S cells with Thal, IMiD1, IMiD2, and IMiD3 was 32%, 55%, 51%, and 43%, respectively. Forty-six percent of annexin V staining was observed in cultures with Dex, whereas only 22% annexin V–positive cells were present in cultures with media alone. The percentage of annexin V–positive Hs Sultan cells and AS patient MM cells was 4% to 7%, respectively, under all culture conditions and was not increased by Thal or the IMiDs.

Effect of Thal and analogs on p21 expression in MM cell lines and patient cells

We next correlated these distinct biologic sequelae of Thal and the IMiDs with p21 status in MM.1S versus Hs Sultan and patient MM cells. As can be seen in Figure 6A, p21 expression was down-regulated by the IMiDs, as well as by Dex, in MM1.S cells; and IL-6 overcomes this inhibitory effect. In contrast, the IMiDs up-regulated p21 in Hs Sultan cells and patient MM cells. Immunoblotting with anti-tubulin Ab confirmed equivalent protein loading. Wt-p53 was recognized in MM.1S cells, whereas both wt- and mt-p53 were recognized in Hs Sultan cells and patient MM cells (Figure 6B). These studies further support the observation that Thal and the IMiDs can induce either apoptosis or G1 growth arrest in sensitive MM cells, and they are consistent with Thal and IMiD p53-mediated down-regulation of p21 and susceptibility to p53-mediated apoptosis in MM.1S cells, in contrast to induction of p21 and growth arrest in Hs Sultan cells and patient MM cells, conferring protection from apoptosis.

Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells

We have previously characterized signaling cascades mediating MM cell growth and apoptosis, as well as the antiapoptotic effect of

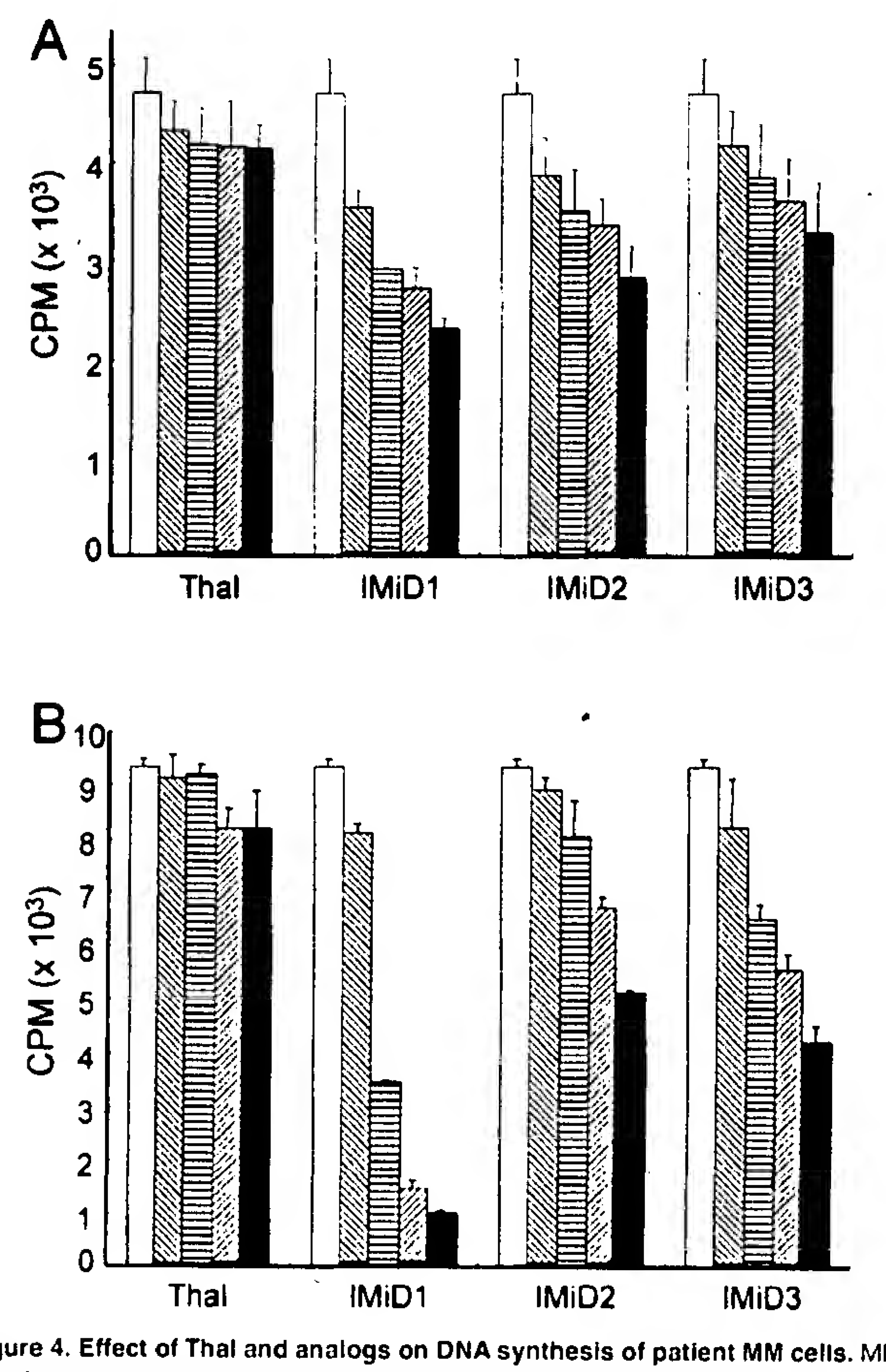

Figure 4. Effect of Thal and analogs on DNA synthesis of patient MM cells. MM cells from patient 1 (A) and patient 2 (B) were cultured with control media (□) or with 0.1 μmol/L (▨), 1.0 μmol/L (▤), 10 μmol/L (▧), and 100 μmol/L (■) Thal, IMiD1, IMiD2, or IMiD3. In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

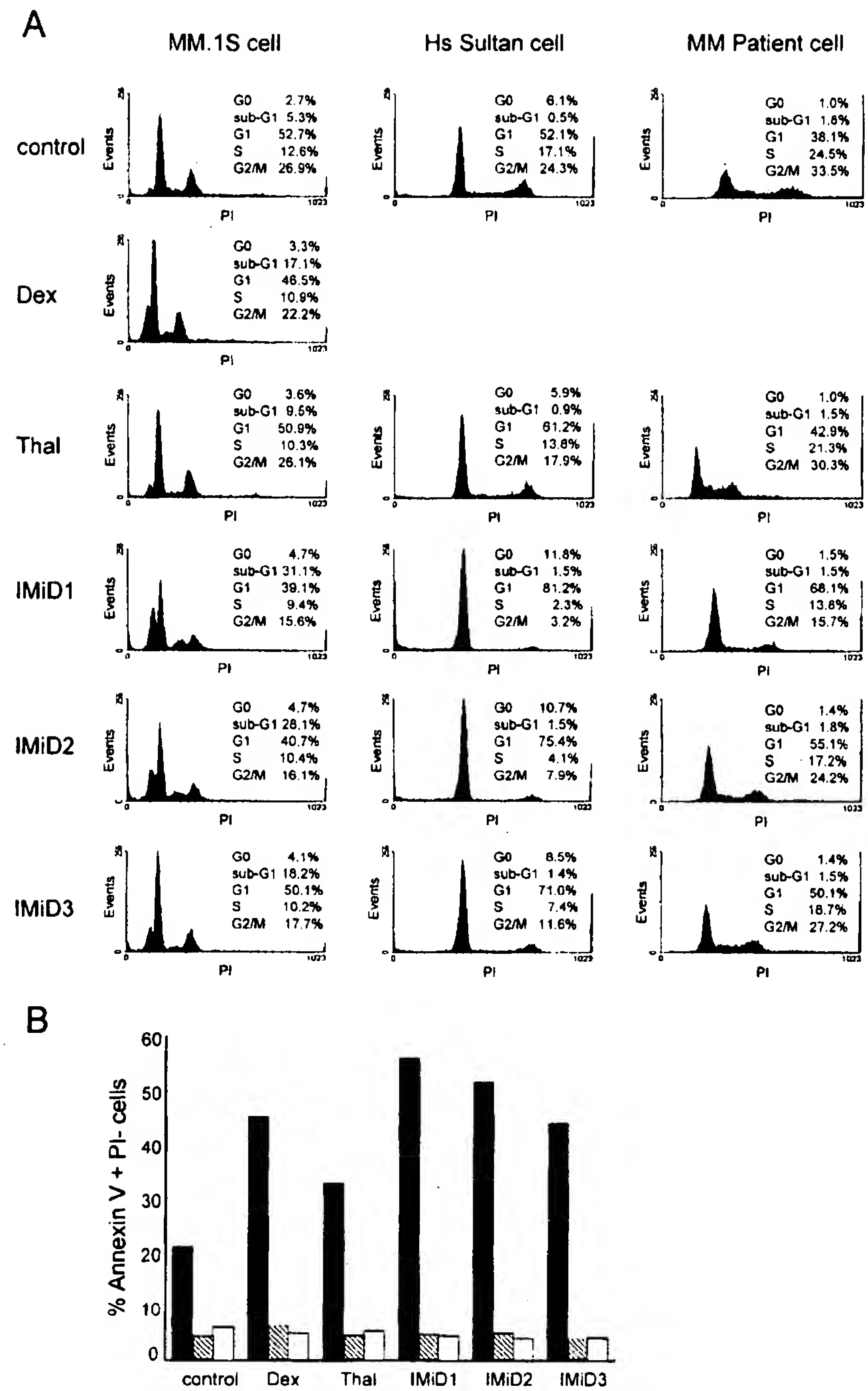

Figure 5. Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells. (A) MM.1S cells, Hs Sultan cells, and patient MM cells were cultured with 10 μmol/L of Thal or 1 μmol/L of IMiD1, IMiD2, or IMiD3 for 72 hours. Cultures in media control alone served as a negative control and 18-hour cultures with 10 μmol/L Dex as positive controls. Cells were then stained with PI, and cell cycle profile was determined by flow cytometric analysis. (B) These MM.1S (■), Hs Sultan (▨), and patient MM (□) cells were also stained with annexin V as an additional assay for apoptosis.

IL-6.[19,22-25] Because we have shown that IL-6–induced proliferation is mediated by the ras-dependent mitogen-activated protein kinase (MAPK) cascade,[19] we next examined the effect of Thal and the IMiDs on tyrosine phosphorylation of MAPK in IL-6–responsive MM.1S cells. Constitutive tyrosine phosphorylation of MAPK in MM.1S cells was down-regulated by the MEK1 inhibitor PD98059 (50 μmol/L), which served as a positive control (Figure 6A), and to a lesser extent by the IMiDs (1 μmol/L; Figure 7A) or Thal (10 μmol/L; data not shown). Treatment of MM.1S cells with IL-6 increased MAPK tyrosine phosphorylation, which was partially blocked by PD98059 but was unaffected by the IMiDs (Figure 7A) or Thal (data not shown). Stripping the blot and reprobing with anti-ERK2 Ab confirmed equivalent protein loading.

The observation that IL-6 can overcome the effects of Thal, the IMiDs, and Dex, coupled with our prior studies delineating signaling cascades mediating Dex-induced apoptosis and the protective effects of IL-6,[22,23,25] suggested that RAFTK activation may be induced during apoptosis triggered by Thal and IMiDs. MM.1S and MM.1R cells were, therefore, next cultured with 1 μmol/L Thal, IMiD1, IMiD2, or IMiD3 for 12 hours. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for activation of RAFTK. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab. As can be seen in Figure 7B, Dex induced tyrosine phosphorylation of RAFTK in MM.1S cells but not in MM.1R cells. Importantly, IMiD1 induced RAFTK tyrosine phosphorylation in both MM.1S and MM.1R

cells, correlating with its effects on both Dex-sensitive and Dex-resistant MM cells.

Discussion

This study demonstrates for the first time a direct dose-dependent effect of Thal and these analogs on tumor cells. Thal has demonstrated clinical anti-MM activity at the University of Arkansas[7] and in this study, and Thal at high concentrations (100 μmol/L) resulted in a modest (< 20%) inhibition of in vitro DNA synthesis of MM cells. SelCIDs also induced a dose-dependent inhibition of MM cells, but only 2 of 4 SelCIDs tested achieved 50% inhibition of proliferation, even at 100 μmol/L concentrations. Importantly, all 3 IMiDs tested achieved 50% inhibition of DNA synthesis at concentrations (0.1-1.0 μmol/L) corresponding to serum levels that are readily achievable, both confirming their direct action on tumor cells and suggesting their potential clinical utility. Moreover, the IMiDs inhibited the proliferation of Dox-, Mit-, and Mel-resistant MM cells by 20% to 35%, and of Dex-resistant MM cells by 50%. These in vitro effects correlate with the observed clinical activity of Thal in patients with MM that is refractory to conventional therapies, both at the University of Arkansas[7] and reported in this study, and suggest their clinical utility to overcome drug resistance. Moreover, our studies further suggest that Dex can add to the antiproliferative effect of Thal and the IMiDs in vitro, suggesting the potential utility of coupling these agents therapeutically. Finally, our study also identified MM cells resistant to Thal and the analogs (U266 cells), which, therefore, can be used to study mechanisms of Thal resistance.

Our studies demonstrate that Thal and the IMiDs are acting directly on MM cells, in the absence of accessory BM or T cells. It is also possible that these agents may be mediating their anti-MM effect by cytokines, given their known inhibitory effects on TNF-α, IL-1β, and IL-6.[15] Our prior studies have characterized the growth

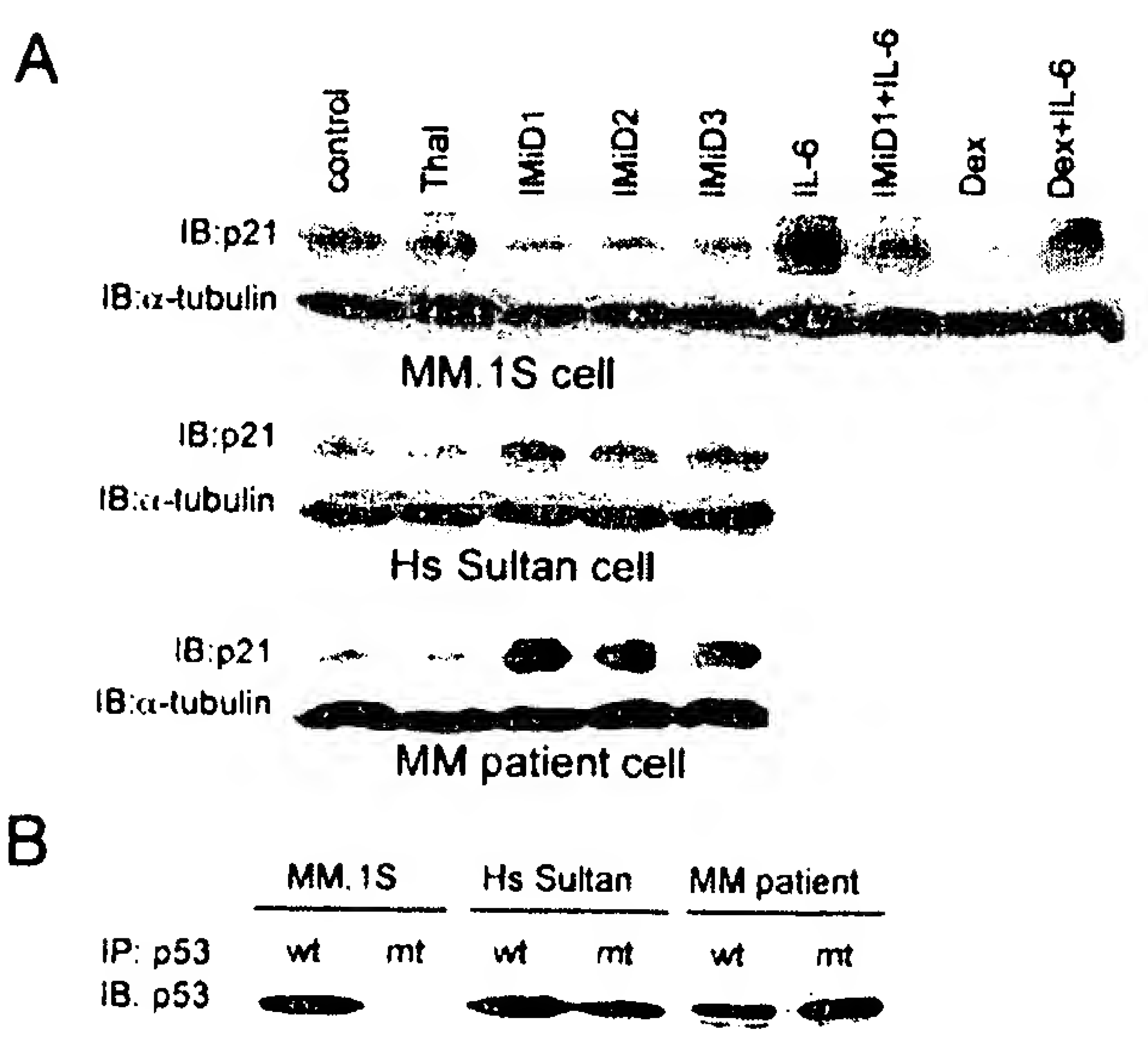

Figure 6. Effect of Thal and analogs on p21 expression in MM cell lines and patient cells. (A) MM.1S cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, and IMiD3 for 48 hours. MM.1S cells were also cultured with IL-6 (50 ng/mL) alone and with IMiD1, 10 μmol/L Dex, and Dex plus IL-6. Cells were lysed, subjected to SDS-PAGE, transferred to PVDF membrane, and blotted with anti-p21 Ab. The membrane was stripped and reprobed with anti-α-tubulin Ab. (B) MM.1S, Hs Sultan, and patient MM cells were lysed and immunoprecipitated with wt-p53 and mt-p53 Abs, transferred to PVDF membrane, and blotted with anti-p53 Ab.

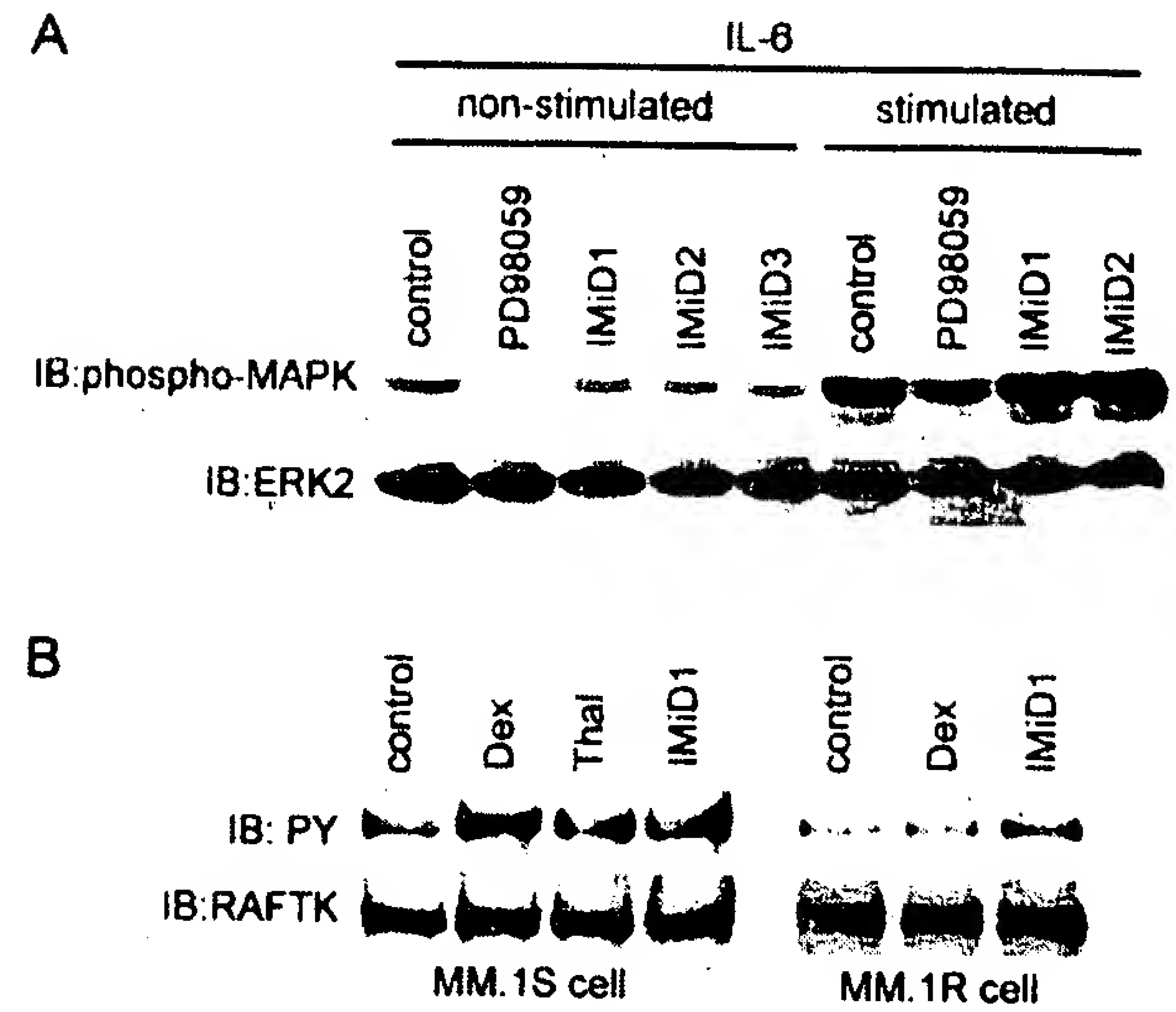

Figure 7. Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells. (A) MM.1S cells were cultured in media, with 50 μmol/L of PD98059 and with 10 μmol/L of IMiD1, IMiD2, or IMiD3 for 48 hours. Cells were then triggered with 50 ng/mL of IL-6 for 10 minutes, lysed, transferred to PVDF membrane, and blotted with anti-phospho MAPK Ab. Blots were stripped and reprobed with anti-ERK2 Ab. (B) MM.1S and MM.1R cells were treated with Thal (100 μM), IMiD1 (100 μmol/L), or Dex (10 μmol/L) and harvested at 12 hours. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab.

effects of IL-6 on human MM cells,[12,26] and we, therefore, next determined the effect of exogenous IL-6 on drug activity. Our studies showed that IL-6 can overcome the effect of Thal and the IMiDs on MM cell lines and patient cells, suggesting that these novel drugs may, at least in part, be inhibiting IL-6 production. Our prior studies have further demonstrated that IL-6–induced proliferation of MM cells is mediated through the MAPK cascade and that blockade of this pathway with either MAPK antisense oligonucleotide or the MEK1 inhibitor PD98059 can abrogate this response.[19,21,24] The present study showed constitutive MAPK phosphorylation in MM cells that is inhibited by PD98059 and, to a lesser extent, by the IMiDs. Importantly, IL-6–triggered MAPK tyrosine phosphorylation is also blocked by PD98059 but not by IMiDs. These studies, therefore, suggest that the IMiDs do not work only by directly inhibiting MAPK growth signaling and further support their potential activity in down-regulating IL-6 production. In MM, IL-6 production in tumor cells can either be constitutive or induced, mediating autocrine tumor cell growth.[26,27] In addition, IL-6 is also produced by BM stromal cells in MM, a process that is up-regulated by tumor cell adhesion to BM stromal cells, with related tumor cell growth in a paracrine mechanism.[10,11] Our ongoing studies are, therefore, evaluating the effect of Thal and these analogs on IL-6 production in the BM microenvironment.

Having shown the inhibitory effects of Thal and the IMiDs on ^{3}H-TdR uptake of tumor cells, we next examined their effect on MM cell cycle. Interestingly, these drugs had distinct functional sequelae in MM cells. Specifically, the IMiDs, and to a lesser extent Thal, induced apoptosis of MM.1S cells, evidenced both by increased sub-G1 cells on PI staining and increased annexin V–positive cells. In these cells that have wt p53, these agents (and Dex) down-regulate p21, thereby facilitating G1-to-S transition and susceptibility to apoptosis. This apoptotic effect may correlate with the clinical observation that complete response to Thal is rarely observed. IL-6 overcomes the down-regulation of p21 induced by these agents, consistent with the increase in DNA synthesis triggered by IL-6 even in the presence of these drugs. In

contrast, in Hs Sultan cells (wt and mt p53) and patient cells (wt p53 and mt p53), the IMiDs and Thal induce p21 and related G1 growth arrest, thereby conferring protection from apoptosis, as has been observed in other systems.[28,29] In our prior study,[20] p21 was also constitutively expressed in the majority of MM cells and also inhibited proliferation in both p53-dependent and -independent mechanisms. Previous reports that cells overexpressing p21 protein demonstrate chemoresistance[30] further support the protective effect of G1 growth arrest induced by these agents in Hs Sultan MM cells and patient MM cells. Conversely, the frequent regrowth of progressive MM noted clinically on discontinuation of Thal treatment may correlate with release of drug-related G1 growth arrest. An ongoing clinical trial is correlating response to Thal with laboratory parameters (ie, serum IL-6 or the surrogate marker C reactive protein) and will gain further insights into its mechanisms of in vivo anti-tumor activity.

Finally, our prior studies have characterized apoptotic signaling cascades in MM, as well as the protective effect of IL-6, especially against Dex-induced apoptosis.[22,23,25,31] Specifically, we have shown that Dex down-regulates growth kinases, such as MAPK and $p70^{RSK}$;[23] importantly, it activates RAFTK, which is required for Dex-induced apoptosis and abrogated by IL-6.[22] The current studies show that IMiD1 acts similarly to Dex, because it activates RAFTK and apoptosis in MM.1S cells, sequelae that are blocked by IL-6. Given our prior studies, which demonstrate that apoptosis of MM cells induced by UV irradiation, γ irradiation, and Fas ligation do not involve RAFTK,[22] the current signaling studies, therefore, further support both the ability of the IMiDs to act through distinct signaling cascades to overcome drug resistance, as well as the enhanced anti-tumor activity observed when Thal or the IMiDs are coupled with Dex.

In conclusion, the results of this study, therefore, demonstrate evidence for direct activity of Thal and the IMiDs against human MM cells. To confirm their in vivo mechanism of action, these compounds and SelCIDs will be examined in an animal model. Importantly, these studies provide the framework for the development and testing of a new biologically based treatment paradigm that uses these novel agents, either alone or together with conventional therapies, to target both the tumor cell and its microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease.

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CU 2546

Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients

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This report of a phase 2 trial of thalidomide (THAL) (200 mg/d; 200 mg increment every 2 weeks to 800 mg) for 169 patients with advanced myeloma (MM) (abnormal cytogenetics (CG), 67%; prior autotransplant, 76%) extends earlier results in 84 patients. A 25% myeloma protein reduction was obtained in 37% of patients (50% reduction in 30% of patients; near-complete or complete remission in 14%) and was more frequent with low plasma cell labeling index (PCLI) (below 0.5%) and normal CG. Two-year event-free and overall survival rates were 20% ± 6% and 48% ± 6%, respectively, and these were superior with normal CG, PCLI of less than 0.5%, and β_2-microglobulin of 3 mg/L. Response rates were higher and survival was longer especially in high-risk patients given more than 42 g THAL in 3 months (median cumulative dose) (landmark analysis); this supports a THAL dose-response effect in advanced MM. (Blood. 2001;98:492-494)

Introduction

Thalidomide (THAL) represents the first new class of active agents in the treatment of multiple myeloma (MM) since the introduction of melphalan and glucocorticoids more than 3 decades ago.[1] Its possible antitumor mechanisms in MM include a direct effect on MM and/or bone marrow stromal cells,[2] modulation of MM stromal cell adhesion,[3] suppression of MM cell–sustaining cytokines,[4] antiangiogenic effects by repression of vascular endothelial growth factor and basic fibroblast growth factor pathways,[5] and immunomodulation such as induction of T_{H1} T-cell response with secretion of interferon-γ and interleukin-2.[6] More recently, synergistic apoptotic signaling of THAL and dexamethasone has also been observed.[7]

We now report on the follow-up of all 169 patients enrolled in a phase 2 trial for advanced and refractory MM.

Study design

Between December 1997 and December 1998, 169 consecutive eligible patients with extensively pretreated and progressive MM were enrolled in a phase 2 trial. THAL (50-mg capsules) (Celgene, Warren, NJ) was started at a daily dose of 200 mg and escalated by 200 mg every 2 weeks to 800 mg according to tolerance. Patients with cardiopulmonary or renal dysfunction were not excluded; liver function tests could not exceed twice the upper limit of normal. All patients were enrolled at a single institution and provided written informed consent in keeping with institutional and Food and Drug Administration guidelines.

Baseline and follow-up laboratory tests were performed as previously outlined.[1] Patients kept a diary to document the occurrence and severity of toxicities. Follow-up visits were scheduled every 3 months, and more than 90% of patients adhered to this.

Study endpoints included paraprotein responses (PPRs) in serum and/or urine of at least 25%, 50%, 75%, or 90%; complete remission (CR) was defined by absence of monoclonal protein on immunofixation analysis.[1] Patients with a PPR less than 25% and those discontinuing treatment before response could be assessed (minimum of 4 weeks of therapy) were considered to have failed treatment; all results were evaluated on an intent-to-treat basis. Relapse criteria have been previously reported.[1]

Survival distributions (Kaplan-Meier) were compared by means of the log-rank test.[8,9] Multivariate modeling of bivariate responses was performed by means of logistic regression and stepwise selection methods. Similarly, multivariate modeling of event-free (EFS) and overall survival (OS) employed stepwise selection and proportional hazard regression models.[10] The percentage of change in laboratory measures was calculated from baseline to 90 days post–THAL administration. Wilcoxon rank sum tests were used to compare the percentage-change distributions of patients with 50% or greater reduction in paraprotein levels and of patients with less than 50% reduction.

Results and discussion

Patient characteristics and percentages consisted of the following: age older than 60 years in 40% of patients, β_2-microglobulin (B2M) greater than 3 mg/L in 50%, abnormal cytogenetics (CG) in 67% (deletion 13 in 37%), longer than 5 years of prior therapy in 20%, and longer 2 years of prior therapy in 72%. Seventy-six percent had received at least 1 and 53% had received 2 or more cycles of prior high-dose therapy with stem cell support. THAL could be escalated to 400 mg, 600 mg, and 800 mg in 87%, 68%, and 56% of patients, respectively. No treatment-related deaths were observed; 58% developed toxicities greater than grade 2 which affected the central nervous system in 25% (mainly sedation and somnolence; confusion; depression; tremor), gastrointestinal tract in 16% (mainly constipation; infrequently nausea or vomiting), and peripheral nerves (sensory neuropathy) in 9%. These toxicities

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were related to both intensity and cumulative dose of THAL administered (data not shown). Fewer than 2% of patients developed deep venous thrombosis (Doppler) or cytopenia.

A PPR of 25% was observed in 37% of patients; a PPR of 50% in 30%; and a PPR of 90% in 14% (Figure 1). Of patients exhibiting 25% PPR, 70% achieved that response within 2 months and 90% within 4.5 months. PPRs of 25% were more frequent with normal CG (52% vs 28%; $P = .003$) and with low PCLI (44% vs 10%; $P < .001$). Importantly, 14% of patients experienced their best response ever on THAL. THAL-induced PPRs were associated with significant reductions in bone marrow plasmacytosis and B2M as well as improvement in hemoglobin and uninvolved immunoglobulin M levels (data not shown).

Twenty-four patients remain on study. Reasons for study removal were disease progression in 105 patients, toxicity in 28, and other reasons in 12. With a median follow-up of 22 months among 84 alive patients, 2-year EFS and OS rates are 20% ± 6% and 48% ± 6%, respectively (Figure 1).

On multivariate analysis, EFS and OS were superior with normal CG, PCLI lower than or equal to 0.5%, and B2M lower than or equal to 3 mg/L, permitting distinction of 4 risk groups (see Figure 1). When results were re-examined without CG and PCLI, which are usually not available in the standard practice setting, B2M greater than 3 mg/L and C-reactive protein (CRP) greater than 7 mg/L emerged as key adverse variables for OS and EFS. Better prognosis was not associated with no prior transplant or longer time lapse since transplant.

To evaluate a possible dose effect of THAL on clinical outcome, a 3-month landmark analysis was performed. Patients given more than 42 g THAL in 3 months (median cumulative dose) had a higher response rate (25% PPR) (54% vs 21%; $P < .001$) and superior 2-year survival (63% ± 8% vs 45% ± 13%; $P < .001$); this was especially the case among patients with at least 1 of 3 adverse prognostic features present (Table 1; Figure 1). Responders (25%, 3-month landmark) had superior 2-year EFS and OS rates (34% and 69%, respectively) compared with nonresponders (20% and 47%, respectively; $P < .001$ and $P = .01$, respectively).

These data extend, in twice as many patients with longer follow-up, our earlier observations in 84 patients.[1] Considering the high-risk study cohort, the EFS and OS rates of 26% and 48%, respectively, 2 years after initiation of treatment are impressive. In fact, 38% of patients had received salvage treatment with dexamethasone (32 patients) or combination chemotherapy (dexamethasone and 4-day continuous infusions of cyclophosphamide, etoposide, and cisplatin [DCEP],[11] 33 patients) and progressed when THAL was initiated.

Results similar to ours have since been reported with THAL alone and in combination with dexamethasone.[12-17] Anticipating a THAL dose-response effect in a patient population with such advanced MM, our study called for dose escalation according to tolerance. Indeed, a dose-response effect was apparent in the high-risk subgroup defined by abnormal CG, B2M, and PCLI. However, prospective investigations are needed to determine, separately in early and advanced MM, the optimal THAL dose and schedule.

Table 1. Higher thalidomide dose benefits patients with high-risk disease

No. risk factors*	Thalidomide dose of more than 42 g/3 mo	No. patients	PPR by at least 25%	P	% alive at 2 y	P
≤ 1	yes	55	45	.01	74	NS
	no	36	19		66	
≥ 1	yes	28	43	.02	42	.01
	no	30	13		20	

*Risk factors are β2-microglobulin > 3 mg/L, plasma cell labeling index > 0.5%, and abnormal cytogenetics.

We had previously not observed a consistent antiangiogenic effect of THAL using serial microvessel density measurements of anti-CD34 monoclonal antibody–stained bone marrow biopsies.[1] This may not be surprising since the major effect of an antiangiogenesis agent should be prevention of new microvessel formation rather than destruction of existing blood vessels. Many of the multiple mechanisms already demonstrated in vitro may be operative in different patient subsets or even in MM subpopulations in the same patient.[7] Gene array technology is uniquely suited to unravel the mechanisms of action of THAL and its congeners in vivo.[18]

The virtual lack of myelosuppression makes THAL an ideal drug for combination with cytotoxic agents earlier in the disease. Such trials are currently in progress. Deep venous thrombosis,[19] hypothyroidism, and bradycardia were more frequent in patients randomized to THAL.[20]

In conclusion, THAL has definite activity in refractory MM. Its role in the up-front management of newly diagnosed MM and as maintenance therapy is under investigation. Issues of pharmacokinetics, dose intensity and scheduling, mechanism of action, and drug combinations need to be addressed.[21] Since THAL's activity in MM may involve, among other things, an antiangiogenic mechanism, this malignancy lends itself well to investigation of strictly antiangiogenic agents such as angiostatin and endostatin, shown to possess remarkable antitumor activity in the human severe combined immunodeficiency disease model of MM (J. Epstein, personal communication, May 2000).

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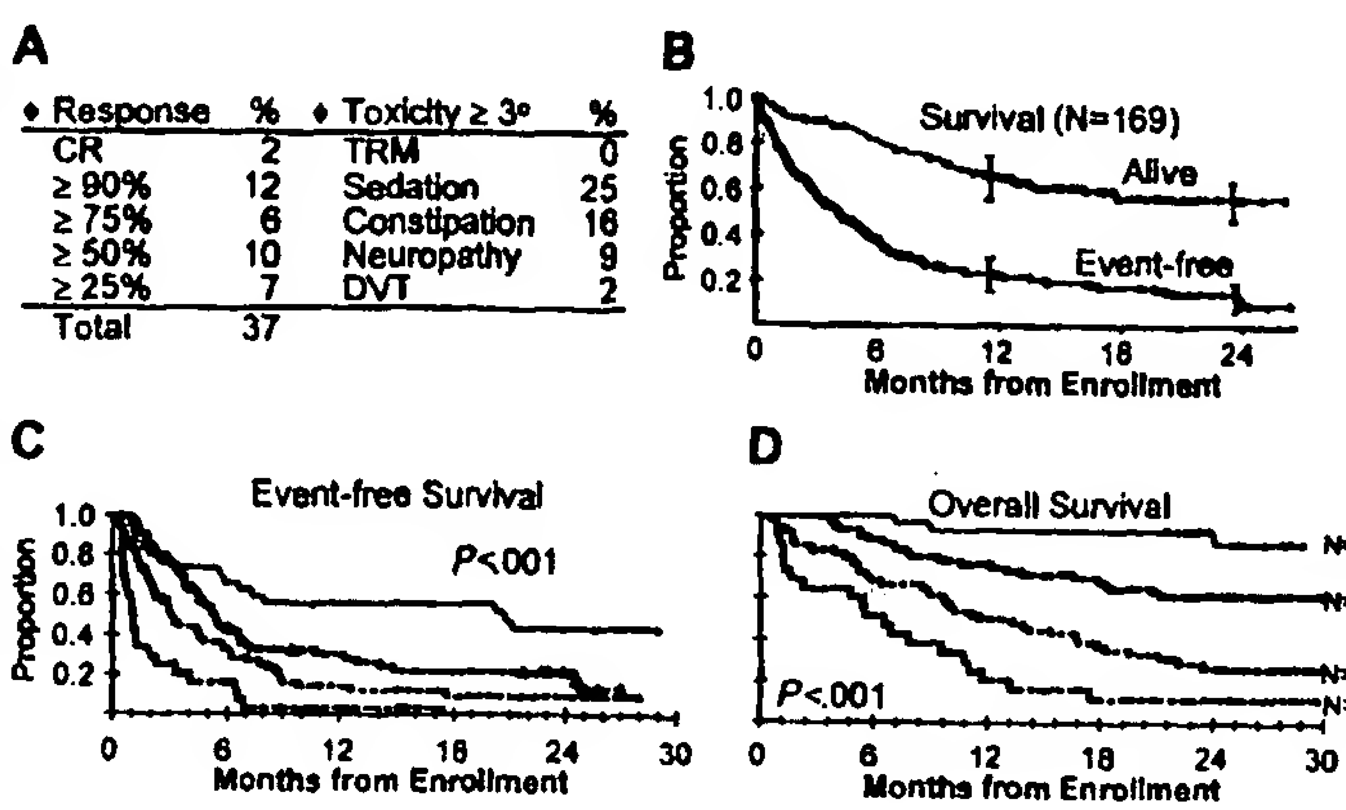

Figure 1. Response, toxicity, and survival. (A) Response rates and grade 3 toxicities; (B) EFS and OS. (C) EFS and (D) OS according to the number of unfavorable prognostic factors present prior to thalidomide. Risk discrimination on the basis of abnormal CG (EFS hazard ratio [HR] 2.15, $P < .001$; OS HR 2.53, $P = .002$); plasma cell labeling index (PCLI) greater than 0.5% (EFS HR 1.86, $P = .002$; OS HR 1.82, $P = .009$); and B2M greater than 3 mg/L (EFS HR 1.54, $P = .016$; OS HR 2.99, $P < .001$). Solid lines indicate no risk factors; dashed line, 1; dotted line, 2; and dash-dotted line, 3 risk factors. Additional unfavorable variables that are only univariately significant included the following for EFS: albumin level less than 3.5 g/dL, $P = .003$; and BM plasmacytosis greater than 30%, $P = .001$. Additional unfavorable variables that are only univariately significant included the following for OS: albumin level less than 3.5 g/dL, $P < .001$; BM plasmacytosis greater than 30%, $P = .05$; hemoglobin level less than 10 g/dL, $P < .001$; creatinine greater than 1.5 mg/dL, $P < .001$; and platelet count fewer than 100 000 µL, $P = .007$. TRM indicates treatment-related mortality; DVT, deep veinous thrombosis.

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INHIBITORS OF ANGIOGENESIS

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Angiogenesis, the formation of new capillaries, is essential to a number of important physiological events, both normal and pathological. Recently, increased attention has focused on the purification and characterization of inhibitors of this process, because of the potential therapeutic value of angiogenesis inhibitors in controlling such "angiogenic diseases" as proliferative retinopathy, solid tumors, rheumatoid arthritis, and neovascular glaucoma. We review the process of neovascularization and the assays that have been developed to study its inhibition *in vivo* and *in vitro*. We also discuss the properties of different angiogenesis inhibitors and examine the mechanisms by which such inhibitors could potentially intervene in the process of neovascularization.

Angiogenesis or neovascularization, the process of new blood vessel formation, is a very tightly controlled process that rarely occurs under normal conditions, except for instances of wound healing, embryonic development and development of the corpus luteum. Otherwise, vascular turnover is very low. It has been demonstrated that the potential doubling time of capillary endothelium from normal tissues is in the range of 47–20,000 days, as opposed to 2.14–13 days for tumor capillary endothelium[1]. In fact, capillary endothelial cells of most normal tissues are considered to be "quiescent".

However, there exist a growing number of diseases (Table 1) characterized by the pathological growth of new capillaries, which are now considered to be "angiogenic diseases"[2]. These diseases include solid tumors, rheumatoid arthritis, psoriasis and a large number of eye diseases including the proliferative retinopathies, neovascular glaucoma, ocular tumors, e.g. retinoblastoma, as well as a large number of diseases associated with corneal vascularization. For example, some 20 other eye diseases are associated with choroidal neovascularization[3], more than 40 diseases are associated with iris neovascularization[4] and retrolental fibroplasia and uveitis are also considered to be angiogenesis-associated diseases.

What is/are the mechanism(s) by which angiogenesis is controlled? In light of the existence of such a large number of diverse angiogenesis stimulators (for review see ref. 5), it is striking that neovascularization rarely occurs normally. There is an accumulating body of literature which suggests that in addition to growth factors, inhibitors of angiogenesis are key regulators of vascular growth.

The availability of a chemical agent which could prevent the continued spread of vascularization would potentially have broad applicability as a therapy for those diseases in which neovascularization plays a prominent role. For example, in nearly all of the eye diseases mentioned above, once neovascularization occurs, the current methods of treatment are often inadequate to prevent further vascular proliferation. Usually, the first line of treatment is directed to the underlying condition and may involve antibiotics, steroids, etc. As vascularization progresses, cautery or photocoagulation is often utilized. At further stages, this type of therapy is increased in frequency and dosage but has often not been satisfactory. Another clinical arena in which an inhibitor of angiogenesis could play a critical role is the control of solid tumors, since tumor growth is angiogenesis-dependent[6] and tumor angiogenesis has been shown to be permissive for metastasis[7]. It has been suggested that an angiogenesis inhibitor could be administered after excision of a primary tumor to prevent metastatic foci from becoming vascularized or used against primary tumors, in particular against highly vascularized yet inoperable tumors (e.g. brain tumors). Additionally, these inhibitors might be used as an adjunct to chemotherapy or immunotherapy[8].

Folkman 1972

THE ANGIOGENIC PROCESS AND *IN VIVO* ASSAYS FOR ANGIOGENESIS INHIBITORS

In the early 1970's, a number of *in vivo* angiogenesis assays had been developed and were routinely used. These model systems included the rabbit corneal pocket, the chick chorioallantoic membrane (CAM), the rat dorsal air sac and rabbit ear chamber (for further review see ref. 7). Critical to the use of many of these assays was the development of controlled release polymers capable of releasing large molecules such as angiogenesis stimulators and inhibitors[9]. The two most commonly used *in vivo* assays are the rabbit corneal pocket model and the CAM assay. In the first assay, polymer pellets of ethylene vinyl acetate (EVAc) copolymer are impregnated with test substance[10] and surgically implanted in a pocket in the rabbit

TABLE 1. Angiogenesis dependent diseases.

1. Angiofibroma
2. Arteriovenous malformations
3. Arthritis
4. Atherosclerotic plaques
5. Corneal graft neovascularization
6. Delayed wound healing
7. Diabetic retinopathy
8. Granulations—burns
9. Hemangioma
10. Hemophilic joints
11. Hypertrophic scars
12. Neovascular glaucoma
13. Nonunion fractures
14. Osler-Weber Syndrome
15. Psoriasis
16. Pyogenic granuloma
17. Retrolental fibroplasia
18. Scleroderma
19. Solid tumors
20. Trachoma
21. Vascular adhesions

From ref. 51.

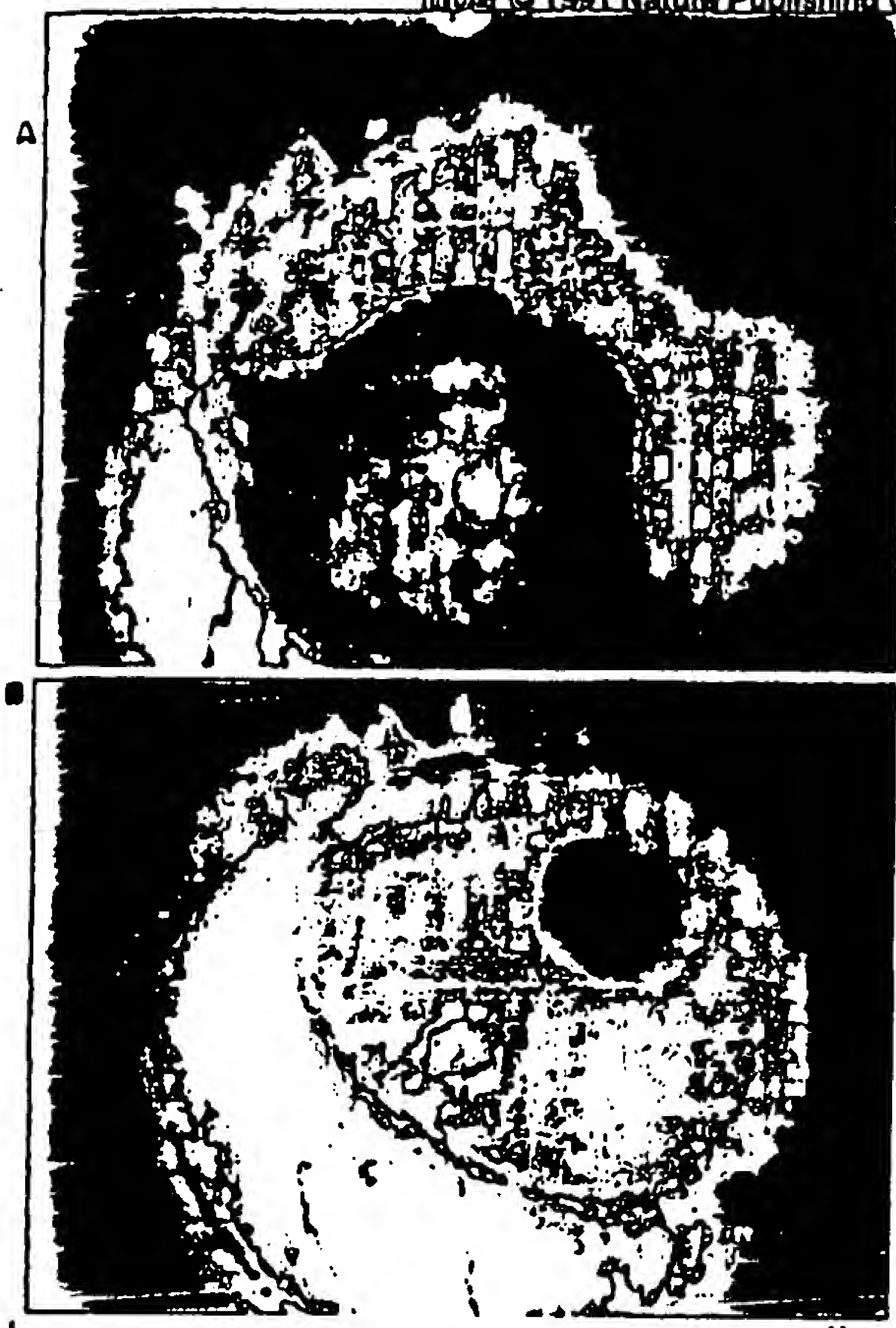

FIGURE 1 Inhibition of tumor-induced angiogenesis by CDI[11] in the rabbit corneal pocket assay. (A) Control cornea implanted with empty EVAc polymer pellet juxtaposed between the limbus of the eye and the V2 carcinoma implant. Capillaries appear as a thick carpet sweeping over polymer and tumor. (B) Test cornea implanted with EVAc polymer pellet impregnated with CDI and juxtaposed between the limbus and the V2 carcinoma implant. Test corneas showed significant inhibition of vessel growth towards the tumor.

cornea approximately 1 mm from the limbus. When this assay system is being used to test for angiogenesis inhibitors, either a piece of V2 carcinoma or some other angiogenic stimulant is implanted distal to the polymer, 2 mm from the limbus. In the opposite eye of each rabbit, control polymer pellets that are empty are implanted next to an angiogenic stimulant in the same way. In these control corneas, capillary blood vessels start growing towards the tumor implant in 5–6 days, eventually sweeping over the blank polymer. In test corneas, the directional growth of new capillaries from the limbal blood vessels towards the tumor occurs at a reduced rate and is often inhibited such that an avascular region around the polymer is observed (Fig. 1). This assay is quantitated by measurement of the maximum vessel lengths with a stereoscopic microscope.

In the CAM bioassay, fertilized chick embryos are placed into Petri dishes and cultured in a humidified incubator in 5% CO_2. On day 6 of development, methylcellulose (1% w/v in distilled water) discs impregnated with the test sample or an appropriate control substance are placed onto the vascular membrane at its advancing edge. On day 8, the area around the implant is observed and evaluated. Avascular zones surrounding the test implant indicate the presence of an inhibitor of embryonic neovascularization[11].

In vivo assays such as these, however, are extremely time consuming, expensive and require large amounts of precious test material. The development of techniques to culture endothelial cells[12] made possible the development of *in vitro* assays (see next section) which could function as "screens" for *in vivo* events. These assays both qualitatively and quantitatively measured the cellular and biochemical components of angiogenesis in a more rapid and reproducible manner using small amounts of test materials.

It is now established that the phenomenon of angiogenesis occurs via the "sprouting" of new capillaries from the preexisting microvasculature. Enzymatic degradation of the basement membrane of the parent microvessel is followed by capillary endothelial cell (EC) migration in response to an angiogenic stimulus. The migrating EC align themselves to form a new sprout. Trailing EC proliferate, thereby increasing the immature sprout length. Subsequently, the process of lumen formation occurs in which two hollow sprouts join to form vascular loops. Pericytes and basement membrane components surround the immature capillaries, completing the formation of the mature capillary bed[13].

IN VITRO ASSAYS FOR ANGIOGENESIS INHIBITORS

With the knowledge of the sequence of events required for neovascularization to occur and with the availability of cultured EC, the development and use of *in vitro* assays devised to expedite the discovery of angiogenesis inhibitors focused on the following components of the angiogenic process: degradation of the basement membrane, migration and proliferation of capillary EC and the formation of three dimensional capillary tubes. To the extent that a factor could inhibit one or all of these key cellular/biochemical events *in vitro*, it is then a candidate for further testing in the standard *in vivo* models.

Capillary EC proliferate in response to an angiogenic stimulus during neovascularization. Therefore, an *in vitro* assay using the cells actually involved in the process of angiogenesis was developed to mimic the process of neovascularization *in vitro*[11]. Capillary EC were stimulated to proliferate by acidic fibroblast growth factor (aFGF) a known angiogenic molecule and then challenged with varying concentrations of test substance. Following a three day incubation period, the number of endothelial cells was measured on the basis of the colorimetric measurement of acid phosphatase.[14] Results were supported by electronic cell counting. This assay is particularly useful because it provides the unique opportunity to screen large numbers of samples in a highly sensitive and reproducible manner.

A second critical event required for angiogenesis to occur is the migration of capillary EC through the extracellular matrix towards an angiogenic stimulus. Migration of endothelial cells can be tested *in vitro* using a modification of the Boyden chamber technique[15]. A blind-well Boyden chamber[16] consists of two wells (upper and lower) separated by a porous membrane. The lower wells receive a known concentration of growth factor and the upper wells receive a predetermined number of cells and inhibitor. Cells attached to the upper surface of the membrane migrate through and attach to the lower membrane surface. The membrane is then fixed and stained for counting[17].

Although a number of factors have been studied *in vitro* which can interfere with one or more of the steps required for angiogenesis, it is essential to test these substances using *in vivo* assays to ensure that angiogenesis is truly inhibited. Only those factors which have been shown to inhibit angiogenesis *in vivo* will be discussed below.

INHIBITORS OF ANGIOGENESIS

Tissue-derived inhibitors. Cartilage has been studied as a potential source of an angiogenesis inhibitor because

of its avascularity. Cartilage is a relatively tumor-resistant tissue and the tumor associated with cartilage, chondrosarcoma, is the least vascularized of all solid tumors. A number of different groups have shown that cartilage and extracts of cartilage inhibit angiogenesis *in vivo* and endothelial cell proliferation *in vitro*[10,18-22]. Partially purified extracts of cartilage have also been shown to inhibit tumor-induced neovascularization when delivered regionally (via controlled release polymer) and when delivered systemically (via infusion)[22]. The critical role that proteolytic enzymes appear to play in the process of neovascularization suggested that protease inhibitors might be anti-angiogenic agents[21-24]. Recently, an angiogenesis inhibitor from bovine scapular cartilage has been purified, characterized and amino terminal sequence data obtained[11]. This molecule, which is an acid and heat stable protein, is a collagenase inhibitor with a relative molecular mass (Mr) of 27,650.

Purified cartilage-derived inhibitor (CDI) is a powerful inhibitor of aFGF-stimulated capillary EC proliferation. Using the assay system described above for measuring capillary EC proliferation, CDI, at a concentration of 96 nM, caused 72% inhibition of proliferation. These results were supported by electronic cell counting assays and tritiated thymidine incorporation studies. Using a modification of the Boyden chamber assay, CDI inhibited capillary EC migration with an IC_{50} (the inhibitory concentration at which 50% inhibition is obtained) of 16 nM[11]. It was further tested for its ability to inhibit angiogenesis *in vivo* on the chick CAM. Purified CDI (4 μg samples) in methylcellulose discs was applied to the surfaces of growing CAMs of 6 day old fertilized chick embryos. After a 48-hour exposure of the CAMs to CDI, large avascular zones were observed as opposed to the control CAMs which never developed avascular zones (Fig. 2). CDI (4 μg = 145 pmols) is a powerful inhibitor of neovascularization *in vivo* when compared to the lowest reported doses of previously reported inhibitors discussed below.

Recently, metalloproteinases such as collagenase have been strongly implicated in the process of malignant conversion, that point in cancer progression when tumor cells gain the capacity to invade and metastasize. A correlation has been demonstrated between the acquisition of the malignant phenotype and an increase in the expression of metalloproteinases (Type IV and transin). Additionally, a key metalloproteinase inhibitor, TIMP (tissue inhibitor of metalloproteinase), is now considered to be a tumor-suppressor gene product[25]. It is interesting to note that CDI differs from TIMP isolated from human amniotic fluid (which itself is virtually identical to a human skin fibroblast inhibitor with the exception of one residue difference) in only two amino acids over the first 28 NH_2-terminal residues[11].

Other avascular tissues have also been studied as a potential sources of angiogenesis inhibitors. Vitreous extracts have been shown to inhibit neovascularization *in vivo*, the lowest reported dose being 200 μg, and endothelial cell proliferation *in vitro*[26,27]. The agent(s) responsible for this inhibition remains to be purified and it appears that vitreous may contain at least two inhibitory species, one ≤ 10 kD, the other ≤ 50 kD[26]. Extracts of human and murine lens have been shown to inhibit endothelial cell proliferation in a cell specific and reversible manner[28]. Additionally, bovine corneal extracts have recently been shown to contain an inhibitor of angiogenesis *in vivo*, which appears to be a low (≤10 kD) molecular weight non-peptide inhibitor. Purification and identification of all of these factors has not yet been accomplished[29].

Angiostatic steroids. Angiogenic steroids are a unique group of molecules which inhibit angiogenesis. Early work on the effects of steroids on angiogenesis showed that heparin or heparin fragments with no anticoagulant activity could inhibit angiogenesis *in vivo*, using the CAM assay, at a dose of 50 μg of heparin with 60 μg of hydrocortisone, and could cause tumor tumor regression and significantly inhibit metastases in the presence of cortisone[30]. These results were important in their support of the hypothesis that an antiangiogenic therapy could ultimately influence tumor growth. Subsequent structure-activity studies with steroid analogs demonstrated that the antiangiogenic activity of the heparin-hydrocortisone combination was not a function of the mineralocorticoid, glucocorticoid or other known bioactivities of hydrocortisone and resulted in the term "angiostatic steroid"[31]. Recently, the use of a synthetic heparin substitute, β-cyclodextrin tetradecasulfate administered with angiostatic steroids, has been shown to inhibit angiogenesis at a lowest reported dose of 14 μg of β-cyclodextrin tetradecasulfate with 60 μg of hydrocortisone in the CAM assay. This drug pair has also been shown to inhibit endotoxin-induced angiogenesis in the rabbit corneal pocket assay both when delivered locally and topically[32].

FIGURE 2 Inhibition of embryonic angiogenesis by CDI (cartilage-derived inhibitor) in the chick chorioallantoic membrane assay (CAM). (A) Normal CAM containing empty methylcellulose disk (B) CAM containing a methylcellulose disk impregnated with CDI surrounded by avascular zone.

Protamine. Protamine, a sperm-derived, cationic protein with a molecular weight of 4.3 kD, has been shown to be a specific inhibitor of angiogenesis, inhibiting neovascularization on the CAM at a dose of approximately 50 μg. When administered systemically, it inhibited tumor growth and metastases in a number of animal models although its efficacy is limited by its toxicity at high doses[33].

Platelet factor-4. Platelet factor-4 (PF4), a collagenase inhibitor with a very strong affinity for heparin, is a tetrameric polypeptide with a molecular weight of approximately 30 kD. It is released from platelets during aggregation as a complex with chondroitin sulfate. This protein was shown to inhibit angiogenesis in the CAM assay at a dose of approximately 10 μg[33,34]. Recent studies using

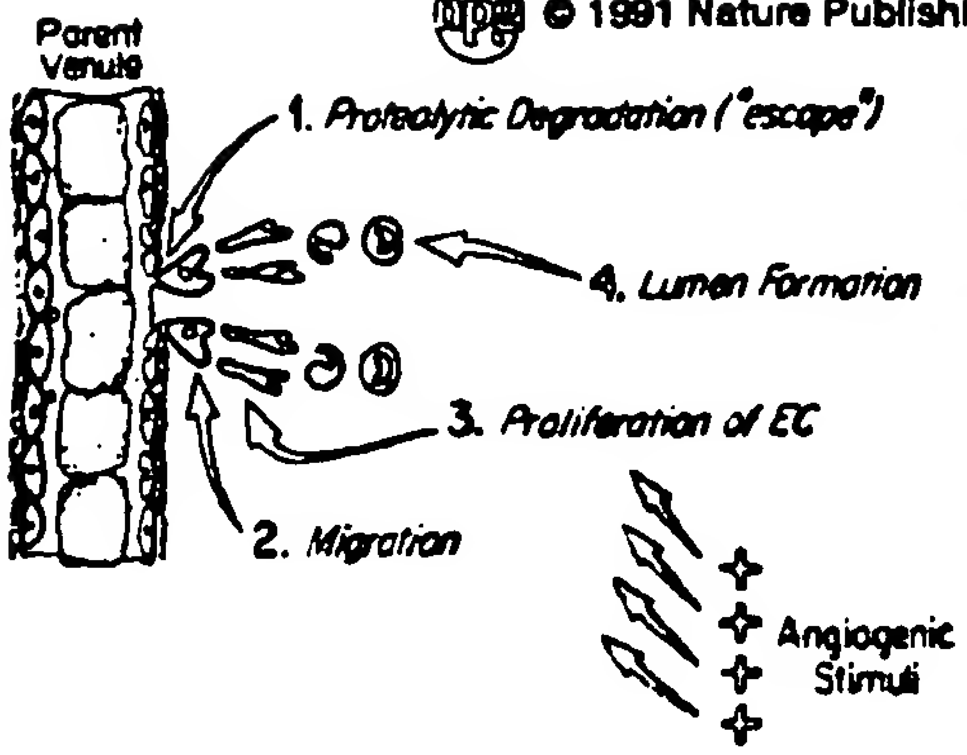

FIGURE 3 Potential intervention sites for the inhibition of neovascularization.

recombinant PF4 have further shown that it is an inhibitor of human umbilical vein endothelial cell proliferation with an IC_{50} of approximately 1 μM[34]. More recently, PF4 has been shown to inhibit the growth of murine melanoma and human colon carcinoma[35].

Thrombospondin. Recently, a 140 kD protein (Gp140) homologous in sequence and function to the C-terminus of human thrombospondin has been shown to inhibit angiogenesis *in vivo* and capillary endothelial cell migration *in vitro*[36,37]. This inhibitory factor was purified from the conditioned media of hamster and human hybrid lines when they were expressing an active cancer suppressor gene. Since the onset of angiogenesis and tumorigenesis was concomitant with the loss of GP140 activity, the production of such an angiogenic inhibitor may be a means by which tumor growth is controlled under certain circumstances[36,37]. These results are consistent with those observed for TIMP, which demonstrate that 3T3 cells which are down modulated for TIMP expression acquire the capacity for invasion and metastases[25].

Other angiogenesis inhibitors. There are a number of other angiogenesis inhibitors that can play a role in negatively modulating neovascularization. These include tumor necrosis factor-alpha (TNF-α), which has been shown to inhibit capillary and aortic cell proliferation as well as smooth muscle growth[38]. Paradoxically, TNF-α stimulates angiogenesis *in vivo*. This effect is similar to that observed for transforming growth factor beta (TGF-β) which, although it is a potent inducer of capillary tube formation *in vitro* and stimulates angiogenesis *in vivo*, is an inhibitor of aortic and capillary endothelial cell proliferation and migration *in vitro*[39,40]. Since angiogenesis can be thought of as a two stage process, one proliferative and one based on differentiation of capillary endothelial cells, it has been hypothesized that TGF-β may affect the process of neovascularization by shifting the balance towards the differentiation of capillary ECs into capillaries, accomplishing this by inhibiting their proliferation and migration[5].

Alpha interferon has been shown to inhibit endothelial cell proliferation and capillary tube formation *in vitro* and has been used as a positive therapy in patients with hemangioendotheliomas[41]. Gamma interferon is also an inhibitor of growth-factor stimulated endothelial cell proliferation as well as a number of transformed cell lines[42]. Certain antibiotics are also anti-angiogenic. Minocycline, a semisynthetic tetracycline antimicrobial with anti-collagenase properties has recently been shown to inhibit tumor-induced angiogenesis *in vivo*[43], and a family of angiogenesis inhibitors (angioinhibins), which are synthetic analogues of fumagillin, have been synthesized and shown to suppress the growth of a wide variety of solid tumors[44].

MECHANISMS OF ACTION

A potential model for the sequence of events involved in angiogenesis is one in which an angiogenic factor(s) stimulates capillary EC to produce proteolytic enzymes such as collagenase and plasminogen activator, which degrade the basement membrane of the parent venule facilitating the release of the capillary EC from the preexisting vessel. In the case of tumor angiogenesis, it has been suggested that tumor cells might release, in addition to angiogenic factors, a chemoattractant for mast cells[45]. Mast cells might then migrate towards the tumor where they release heparin, a glycosaminoglycan, which has been shown to enhance the activity of angiogenesis factors *in vivo*[33,46] and which can potentiate growth-factor stimulated EC proliferation and migration *in vitro*[46,47]. The capillary cells, under the influence of angiogenic factors and heparin, which has also been shown to stimulate collagenase activity in EC[48], would then migrate out of the parent vessel and into the perivascular space and through the local extracellular matrix (facilitated by protease activities) towards the angiogenic stimuli. These cells, which have "sprouted" from the parent vessel, proliferate in response to angiogenic stimuli and begin to elongate the capillary sprout. Finally, these capillary EC join to form lumen and eventually, with branching, form a mature capillary network surrounded by basement membrane[15]. The inhibition of angiogenesis may be accomplished via a number of different biochemical and cellular means all focused on intervening in the process of neovascularization at these key junctures (Fig. 3).

One early event in neovascularization is the breaching of the parent venule's basement membrane to allow EC "escape" and the subsequent capillary EC migration through extracellular matrix. At this stage and throughout the process of capillary formation, EC must degrade extracellular matrix in order to move towards the angiogenic stimulus. Collagenase has been shown to be one of the key proteases required at these stages of neovascularization[28]. Additionally, collagenase has been shown to be required for tumor cells to breach the boundaries of the vascular tree during metastases[24]. The fact that the collagenase inhibitors CDI, PF4 and minocycline are anti-angiogenic is consistent with earlier work demonstrating the importance of collagenase in neovascularization and suggests that at least one way in which they exert their inhibitory effects on neovascularization might be through inhibition of this key proteolytic enzyme.

Furthermore, both CDI and PF4 ultimately cause local perturbations in the extracellular matrix, that is, they can cause changes in the integrity and/or quantity of intact basement membrane components, in this case, collagen. These changes have been shown to play a role in the control of neovascularization, since normal capillary development requires an intact basement membrane[49]. Other inhibitors such as the angiostatic steroids may work through similar mechanisms. The angiostatic steroids have been suggested to act to inhibit neovascularization by altering capillary basement membrane turnover when administered with heparin[49].

Another potential mechanism for anti-angiogenic activity focuses on the glycosaminoglycan, heparin, and its ability to enhance the effect of angiogenesis factors. For example, protamine has been shown to possess a very strong affinity for heparin as do many potent stimuli of angiogenesis (heparin-binding growth facors). It has been hypothesized that the anti-angiogenic effect of protamine is a function of its interference with these growth factors' stimulation of capillary EC functions via binding of heparin. In support of this hypothesis, it has been shown that

the mitogenic activity of soluble and matrix-bound FGF is inhibited by protamine[50]. Additionally, PF4 was originally tested for its anti-angiogenic ability due to its high affinity for heparin and it has been suggested that the mechanism by which it inhibits neovascularization may be similar to that of protamine[33].

CONCLUSION

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate[37]. In those rare instances in which neovascularization occurs under normal physiological conditions, such as in wound healing, corpus luteum development and embryogenesis, it is stringently regulated and temporally and spatially delimited[37]. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. These observations suggest that a viable therapeutic strategy for the control of those diseases characterized by deregulated neovascularization can be developed pending the availability of potent, reliable and biologically compatible angiogenesis inhibitors.

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Tumor Growth and Neovascularization: An Experimental Model Using the Rabbit Cornea [1,2]

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SUMMARY—By intracorneal implantation of tumors in the eyes of rabbits, the early neovascular response to solid tumor growth could be directly observed. Fragments of homologous tumors—Brown-Pearce epithelioma and V2 carcinoma—were implanted into the avascular corneal stroma of rabbits at various distances from the limbus. Tumor growth and neovascular response of limbal vessels were studied by: 1) Slit Lamp Stereomicroscopy, 2) histologic examination, 3) filling of vasculature with colloidal carbon, and 4) autoradiography after exposure to ^{3}H-thymidine. Centrally placed tumors spread as thin plates until they reached within 2.5±0.5 mm of the limbus, when new vessels began to grow from the limbal plexus toward the tumor edge. When tumors became vascularized, they grew rapidly into exophytic masses. Peripherally placed tumors evoked early neovascularization. The prevascular growth of incompatible rabbit homograft and mouse xenograft tumors suggested that the cornea, before its vascularization, was an immunologically privileged site for tumor growth. Intracorneal polyacrylamide gel implants containing tumor extracts elicited a specific pattern of corneal vascularization not observed with nonmalignant cell extracts. These experiments provide a new model for study of tumor growth and neovascularization in a site where there is anatomic separation of tumor cell stimulus from host vascular response; the technique of corneal gel implantation may be useful in the characterization of mediators of neovascularization.—J Natl Cancer Inst 52: 413–427, 1974.

THE GROWTH of solid neoplasms is associated with the proliferation of capillaries in surrounding host tissues [*see* (*1*) for review]. Studies in this laboratory and other laboratories suggested that the malignant parenchymal cells and the nonmalignant host vascular stroma within a solid tumor are mutually dependent (*1, 2*). Tumor cells can stimulate endothelial cell proliferation and new capillary outgrowth (*3, 4*), without which a tumor implant remains dormant at a small size (*5*). Ideally, if the malignant parenchyma of a solid tumor could be experimentally separated from the responding host vessels, ways of disrupting this interdependence could be better studied.

This report describes the use of the rabbit cornea to study prevascular tumor growth and host's angiogenic response to live tumor cells and tumor-angiogenesis factors (TAF's) (*3, 6*). The cornea provided a transparent avascular substratum in which these events could be continuously observed in vivo. Implantation at varying distances from the circumferential limbal vessels produced an anatomic separation of tumor cells from responding host vessels, which allowed independent observations on the behavior of both elements. Although the cornea has been used extensively to investigate various aspects of neovascularization, to our knowledge, this is the first time the cornea has been used to examine in detail tumor growth and angiogenesis.

The current study was concerned primarily with the vascular events after corneal implantation of 2 homologous rabbit tumors: the Brown-Pearce epithelioma, which spontaneously regresses, and the V2 carcinoma, which grows progressively. Preliminary experiments are also presented on intracorneal gel implants of Walker 256 carcinoma *extracts* with known angiogenic activity (*3, 6*). Microgram quantities of active TAF fractions elicited a distinctive pattern of corneal vascularization, as compared to the pattern produced by nonmalignant cell fractions and inactive fractions of malignant cells.

MATERIALS AND METHODS

Animals.—Male New Zealand White rabbits, initially 6 weeks old and weighing 2–3 kg, were used for propagation of tumor stocks and for all experiments.

Tumors.—Stocks of Brown-Pearce epithelioma[7] and V2 carcinoma[8] were maintained by sterile anterior chamber and intramuscular passage, respectively; 7- to 14-day-old non-necrotic nodules were sterilely excised and minced with iridectomy scissors in isotonic saline.

Implantation technique[9] (*text-fig. 1*).—Intravenous pentobarbital anesthesia (25 mg/kg) was supplemented by retrobulbar infiltration with 2% Xylocaine (text-fig. 1, part 1). The eye was moved forward and secured in position by a fold clamped in the

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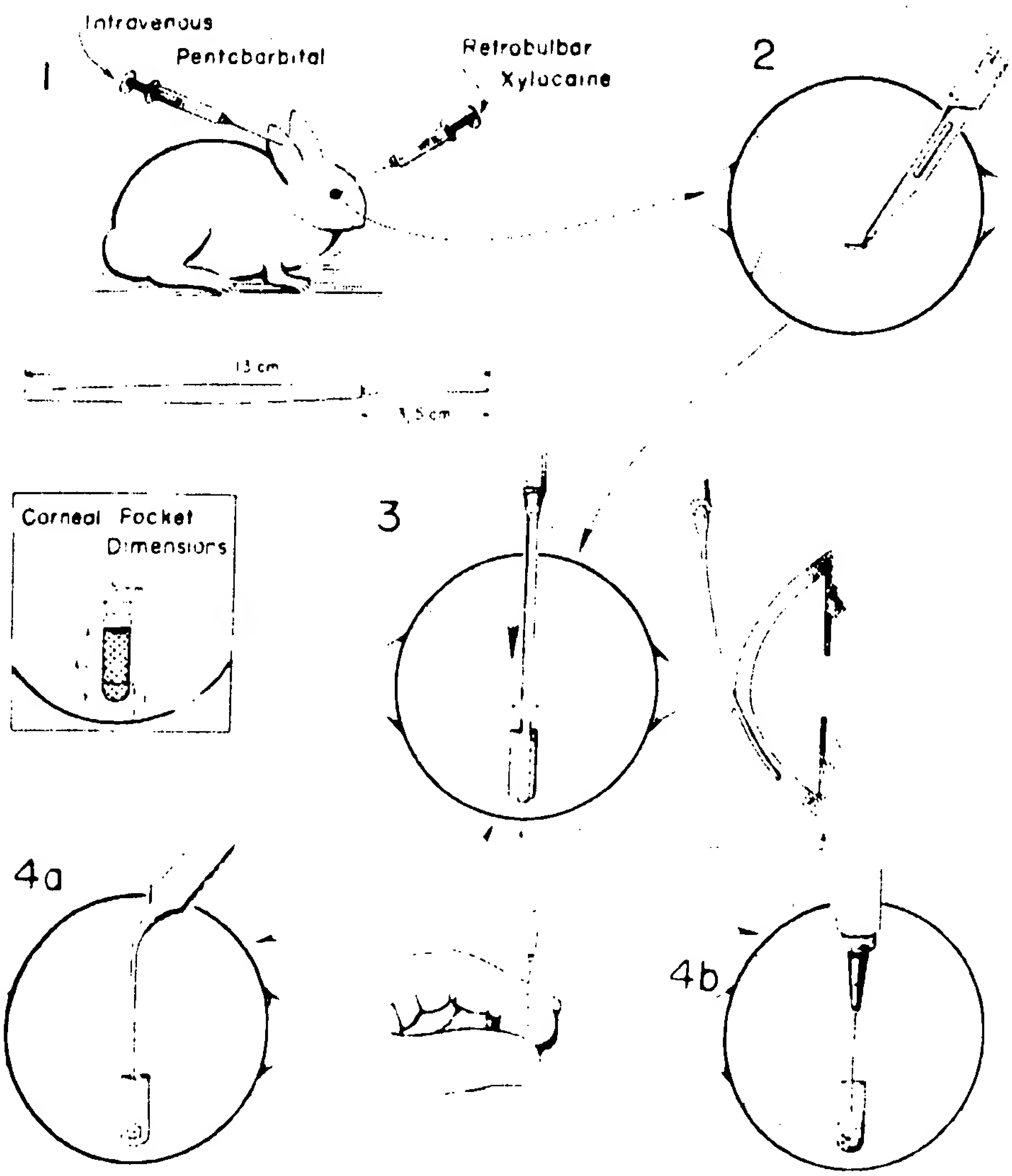
lower lid. With a Bard-Parker #11 blade, a superficial incision, 1.5 mm long, was made in the corneal dome to one side of its center (text-fig. 1, part 2). Before proceeding, we reduced intraocular tension by draining a small amount of aqueous humor from the anterior chamber through a 27-gauge needle. The incision was then continued down into, but not through, the cornea. A malleable iris spatula (1.5 mm width) was inserted and an oblong pocket fashioned within the corneal stroma (text-fig. 1, part 3). Peripheral pockets ended 1–2 mm from the limbus (cornea-scleral junction); central pockets were positioned near the corneal apex (5–7 mm from the limbus). A small (about 1 mm^3) piece of minced tumor was deposited in the bottom of each pocket (text-fig. 1, part 4a), which then sealed spontaneously. When an inadvertent perforation occurred, the corneal pocket was not used. All procedures were performed sterilely. No antibiotics or special eye care was necessary.

Cell-free extracts of tumor cells and normal cells in polyacrylamide gel.—Each cell-free extract of malignant and normal cells was simultaneously tested for angiogenesis activity in the rat dorsal air-sac assay, as previously described (5). The following extracts were tested: 1) "Crude" cytoplasmic TAF was obtained by nitrogen cavitation of Walker 256 ascites tumor cells. Nuclei were discarded, and the supernatant was centrifuged at 360,000 × g for 1 hour. The pellet contained angiogenesis activity (5). 2) Nuclear chromatin, nonhistone TAF fractions were prepared from Walker 256 ascites tumor cells (6). Their angiogenesis activity was greater than that of crude cytoplasmic TAF on rat bioassay. 3) Nuclear chromatin, histone fractions, similarly isolated from Walker 256 ascites tumor cells (6), contained negligible angiogenesis activity. 4) Nuclear chromatin, nonhistone fractions were prepared from adult rat liver cells by the same technique as that used for TAF preparations (6). These contained no angiogenesis activity on rat bioassay.

In the original experiments (*see* table 1), soft polyacrylamide gel, buffered at pH 7.4, was prepared by the adjustment of the proportions of reagents, worked out in our laboratory by G. Mackie (unpublished data) and described by Folkman et al. (7), to yield a



TEXT-FIGURE 1.—Technique of corneal implantation. 1) Systemic and local retrobulbar anesthesia; 2) perpendicular incision into the corneal stroma (approximately half thickness); 3) intrastromal pocket created with malleable iris spatula; 4) tumor fragment (a) or test extract in polyacrylamide gel (b) inserted into corneal pocket. Peripheral pockets ended 1–2 mm from limbus (corneal-scleral junction); central pockets ended 5–7 mm from limbus.

7% acrylamide solution. All reagents were dissolved in sterile, pyrogen-free, distilled water and were passed through a Millipore filter immediately before use. However, a modification was recently made (R. Arensman, D. Ausprunk, J. Folkman, unpublished data) that reduces nonspecific inflammation due to the acrylamide deposit; i.e., all reagents were dissolved in Ringer's lactate instead of distilled water and the acrylamide solution was made up to 5%, rather than 7% as follows:

Solution A:

Acrylamide	1.30 g
Bisacrylamide	0.055 g
Hepes buffer	0.169 g

Dissolved in 25 ml Ringer's lactate

Solution B:
Ammonium persulfate, $(NH_4)_2S_2O_8$.
1 g in 10 ml Ringer's lactate.

Solution C:
Sodium bisulfite, $NaHSO_3$.
1 g in 10 ml Ringer's lactate.

Solution A was adjusted to pH 7.4–7.6 with 1N NaOH. To 25 ml of solution A, 0.35 ml solution B was added, followed by 0.35 ml solution C. Polymerization occurred after about 10 minutes.

Samples of each extract were added during polymerization of the polyacrylamide to produce a 20% final suspension (vol/vol). The resulting soft gels were easily extruded through a 25-gauge needle from a microliter syringe. A total of 5–10-μl of gel was deposited into peripheral corneal pockets (text-fig. 1, part 4b). Each sample of the cytoplasmic TAF contained about 100–200 μg protein; nuclear TAF and liver cell samples contained about 1.0–2.0 μg protein.

Stereomicroscopic observations.—Eyes with corneal implants were examined daily by 2 observers with the aid of a Zeiss Slit Lamp Stereomicroscope (Carl Zeiss, Inc.), at 10–40×. Tumor and new vessel growth were measured en face with an ocular micrometer at 10× (measurement error ±0.1 mm). A green filter allowed clearer definition of fine vascular channels developing within the cornea; intravenous injection of 0.5 cm³ fluorescein (fluorescite, 5% aqueous solution, Moore Kirk Laboratories) kg and cobalt blue illumination helped to outline objectively areas of perfusion within growing tumors. Serial diagrams of the vascular reactions associated with >100 corneal implants over a 12-month period were thus collected and compared.

Histologic and autoradiographic studies.—Entire anterior segments (cornea and iris) were excised and fixed by immersion in 10% buffered formalin. Paraffin sections were prepared and stained with hematoxylin and eosin or other special stains when indicated (see "Results"). In certain experiments, freshly excised, anterior segments were incubated in vitro at 37° C in medium 199 containing 8 μCi/ml ³H-thymidine (³H-TDR) (sp act, 2 Ci/mM, New England Nuclear Corp.) for 1 hour before fixation. Autoradiographs were then prepared by standard techniques (3).

Colloidal carbon vascular injection.—Patterns of new vessel formation within the cornea were outlined by arterial injection of filtered colloidal carbon (Guenther-Wagner, Pelikan, Hannover, Germany), and glycerin-cleared wholemounts were prepared as described in (9).

RESULTS

Summary of Observations

Two distinct phases of tumor cell growth were observed after corneal implantation of tumor fragments: prevascular and vascular (text-fig. 2). The prevascular phase began when the tumor slowly expanded as a thin plate beyond the pocket boundaries and ended when newly formed corneal vessels reached the periphery of the tumor. These vessels originated as capillary loops from small venules in the adjacent limbal plexus and grew radially, penetrating the corneal stroma in two or more planes. At the point of union with the advancing tumor edge, a rapidly growing mass developed (vascular phase). Behavior following vascularization depended on tumor type: Brown-Pearce epitheliomas eventually regressed, while V2 carcinomas continued to grow into exophytic masses. The duration of the prevascular phase was related to the initial position of the implant, regardless of tumor type: Near the limbus, vascularization occurred early (7–12 days); beyond 3 mm, it was much delayed (3–5 wks).

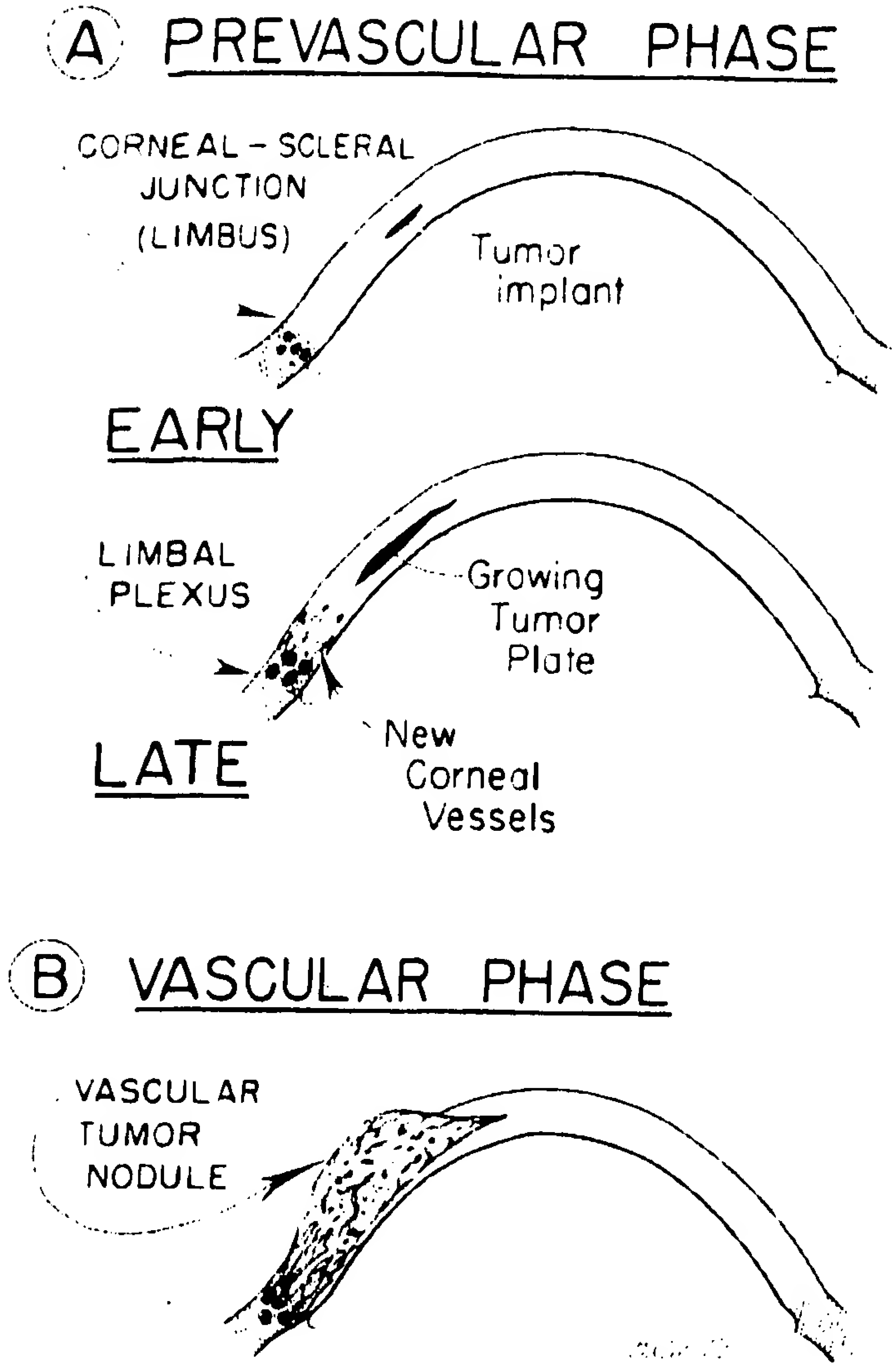
Empty corneal pockets fashioned at distances >1 mm from the limbus never stimulated corneal neovascularization.

Tumor fragments, killed by being boiled in saline for 15 minutes and implanted in peripheral pockets (1–2 mm from limbus), did not elicit any macroscopically detectable changes in the cornea or adjacent limbal vessels. They remained visible, as inert, granular deposits, for as long as 3 months. Similarly, fragments from necrotic areas of older stock tumors often failed to grow and elicited no neovascular response.

Prevascular Phase

Viable tumor fragments began to grow within 24–36 hours after implantation. Centrally placed implants (about 6 mm from the limbus) grew slowly as thin, circular plates within the corneal stroma. The thickness of these implants appeared to remain constant on slit-lamp examination, while growth occurred around the periphery of these implants. The surrounding corneal stroma remained clear, and, at 40×, a feathered edge was visible where the tumor was penetrating among the interdigitating corneal lamellae.

A histologic section of a 35-day-old central implant of Brown-Pearce epithelioma, in its prevascular growth phase, is shown in figure 1a. A broad, thin plate of tumor, 20–30 cells thick at its maximum, was surrounded by relatively normal-appearing corneal matrix. The growing edge tapered to a layer 1–4 cells thick and was well demarcated from the surrounding corneal stroma, which remained free of inflammatory infiltrate (fig. 1b). In the center of the prevascular plate, tumor cells often exhibited degeneration and necrosis, with accumulation of



TEXT-FIGURE 2.—The 2 phases of tumor cell growth and summary of observations with tumor implants.

polymorphonuclear leukocytes. However, the major part of the plate, its growing edge, and the layers of cells adjacent to the corneal stroma appeared histologically viable. Incorporation of ^{3}H-TDR was localized to tumor cells in the periphery of the plates in both tumor types (figs. 2, 3).

The edge of central implants grew slowly toward the limbus at an average rate of 0.10–0.20 mm/day. No capillary sprouts from limbal vessels were observed until this border approached within 2.5 ± 0.5 mm (mean ± SD, 10 implants), usually during the 3d–4th week after implantation. At this stage, histologic sections of the stroma between the tumor edge and limbal vessels were free of inflammatory infiltrate; limbal vessels themselves were occasionally engorged but rarely exhibited neutrophilic exudation (fig. 4).

In contrast, peripheral implants (1–2 mm from limbus) elicited new capillary outgrowth as early as the 4th day after implantation when they had not grown beyond the boundaries of their pockets. The prevascular phase of peripheral implants thus was limited to the 1st week.

Host Neovascular Response

Newly formed vessels always penetrated the cornea centripetally from the adjacent limbal area and grew toward the advancing tumor edge. Delicate capillary loops originated perpendicularly from small venules in the limbal plexus and then grew as elongated hairpins with occasional flame-shaped hemorrhages at their apices. Linear growth of these hairpin vessels initially proceeded at approximately 0.50 mm/day. Then secondary and tertiary branches rapidly developed, converting the initial sprouts into dense, vascular brushes. Vascular proliferation was greatest in the anterior layers of the corneal stroma but also occurred in deeper regions. Histologic sections of growing capillaries approaching the tumor edge showed that the surrounding stroma was essentially free of inflammatory cells; the vessels proper showed slight or no margination and exudation of leukocytes.

Remnants of blood-filled vessels occasionally were visible within recently implanted tumor fragments. However, new vessel outgrowth from tumors into the surrounding corneal stroma was never observed.

Vascular Phase

Penetration of any sector of the tumor plate by corneal vessels was followed by an abrupt increase in tumor volume at that site. Adjacent, thin areas gradually became incorporated into the expanding, vascularized tumor mass (figs. 5, 6). "Feeder" vessels were prominent within the corneal network, and vasodilatation of the parent limbal plexus occurred.

Figure 7 (a, b) illustrates a typical sagittal section through a central implant which was penetrated by corneal vessels along its limbal border; at this point, the broad avascular plate, which developed during the preceding 28 days, had recently grown into a vascularized tumor nodule.

Vascularized implants of V2 carcinoma grew progressively into bulky, exophytic masses which protruded from the corneal surface (figs. 8, 9) and eventually ulcerated. Histologic sections of the growing tumor often revealed foci of necrosis with infiltration by neutrophils and mononuclear cells. Vascular tumor growth proceeded in 3 dimensions at a linear rate of 0.2–0.5 mm/axis/day, with an apparent volume-doubling time of ≤24 hours. In a series of 25 implants of V2 carcinoma, spontaneous tumor regression was never observed.

Recently vascularized implants of Brown-Pearce epithelioma initially exhibited a similar increase in growth rate and transition to a 3-dimensional growth pattern. Figure 6 illustrates the dense, vascular network that developed within the periphery of an implant of Brown-Pearce epithelioma.

However, in each of 20 viable implants of Brown-Pearce tumor, lytic areas began to appear within the tumor mass 5–7 days after vascularization. After 12–14 days, a grossly visible decrease in tumor volume had occurred, which progressed to complete dissolution of the tumor cell parenchyma by the 3d week, as seen in the Slit Lamp Stereomicroscope. The

same time course for tumor lysis was observed after vascularization of both peripheral and centrally placed implants, despite the great difference in the duration of the prevascular growth phases of these implants.

With regression, the dense, neovascular network (fig. 6) gradually assumed a "pruned-tree" outline (fig. 10), in which tertiary and secondary branches disappeared, leaving bare hairpin loops. By the 4th week, flow in these primary channels ceased, as judged by intravenous fluorescein injection.

Histologic sections during regression (figs. 11, 12) revealed an inflammatory infiltrate that was predominantly perivascular and mononuclear. Tumor cells exhibited vacuolization, nuclear condensation, and signs of frank necrosis (fig. 12).

The only remnants of the completely regressed tumor were cords of small clusters of lipid-laden cells that also contained lipofuscin and occasionally hemosiderin pigments. Histologic and ultrastructural details of regressing tumor vessels will be reported separately (D. Ausprunk et al., in preparation).

Xenografts and Homografts in Presensitized Animals

In 6 animals in which intracorneal Brown-Pearce epitheliomas had completely regressed, a second tumor fragment of this type was implanted into the contralateral, normal cornea. Prevascular tumor growth and new vessel formation proceeded as described for primary implants. However, vascular penetration of any sector of the tumor plate was followed by tumor lysis at that point within 24 hours, rather than after the usual period of 5–7 days.

Intracorneal Brown-Pearce implantations were also performed in 3 animals which were presensitized by recent rejection of intramuscular tumors of the same type (*10*). In these animals, prevascular tumor growth and corneal neovascularization proceeded normally, but vascular penetration resulted in immediate lysis of the tumor plate. In contrast, V2 carcinomas implanted into the contralateral corneas in the same animals continued to grow after vascularization.

In 6 instances intracorneal xenografts of fragments of the transplantable B16 mouse melanoma (carried in C57BL/6 hosts) were attempted. After central corneal implantation, thin, pigmented, avascular tumor plates developed. When the tumor edge approached within 2–3 mm of the limbal plexus, a centripetal outgrowth of corneal vessels occurred, similar to that observed with homologous rabbit tumors. Penetration by corneal vessels resulted in immediate lysis of the tumor plate.

Cell-Free Extracts

Polyacrylamide gel suspensions of extracts from malignant (Walker ascites) and normal (rat liver) cells produced 3 general patterns of limbal vessel response, as observed daily with the Slit Lamp Stereomicroscope in vivo and outlined with colloidal carbon at the time the animals were killed.

1) Negative response.—Early limbal engorgement [illegible] or exudate was observed. New vessel growth either did not occur (fig. 13) or there was a slight capillary outgrowth that did not penetrate the area of the pocket (figs. 14, 15) for up to 90 days of observation.

2) Neovascular pattern.—Limbal vessel engorgement (day 3) was followed by fine, hairpin outgrowths (day 5), without signs of gross corneal edema or inflammatory exudate. A dense, vascular outgrowth then progressed at the rate of 0.25–0.50 mm/day toward the pocket edge (day 7–10). Penetration in and around the gel-filled pocket, with arborization and development of a fine capillary network, followed (day 10–16) (figs. 16, 17). Blood flow persisted within this network for several weeks, after which the vascular channels gradually disappeared.

3) Nonspecific inflammatory pattern.—This was characterized by early (day 1–3) appearance of gross corneal edema and moderate-to-severe conjunctivitis and iritis. A dense, white exudate tended to accumulate within the corneal pocket; samples of this exudate failed to yield growth on routine bacteriologic culture. A broad, brush-like pannus of vessels then developed, which remained radially oriented and did not arborize within the pocket (fig. 18) for up to 60 days' observation.

Table 1 summarizes the corneal vascular response to various cell-free preparations implanted in polyacrylamide gels. Angiogenesis activities obtained in simultaneous rat bioassays (*3*) are tabulated for comparison. A high degree of correlation was noted for positive or negative neovascular responses between the 2 systems. Some false-negative responses in the cornea were observed with cytoplasmic TAF (1/7) and nuclear, nonhistone TAF (1/5) fractions. In this series, inflammatory responses occurred in up to 25% of controls. Although such responses can be mistaken for positive responses if daily observations are not made, the data indicate a correlation between the rat bioassay system and the corneal gel implants, even if all responses, inflammatory or positive, are counted as positive.

In more recent experience, when Ringer's lactate was used as the solvent for polyacrylamide instead of distilled water, inflammation was reduced, but still occurred to varying degrees with different lots of acrylamide reagents.

DISCUSSION

Corneal implantation provides an experimental model for tumor angiogenesis studies. It has several useful features: 1) Tumor growth and host vascular response are initially separated, which allows independent observations of the behavior of both elements, 2) the cornea is a naturally transparent, avascular structure, in which the rates of tumor growth and new vessel formation can be easily measured; and 3) the Slit Lamp Stereomicroscope enables frequent in vivo observations, without special manipulations, in an unanesthetized animal. The natural "chamber" of the cornea permits us to observe prevascular tumor growth and the earliest angiogenic response to live tumor cells and TAF's.

TABLE 1.—*Corneal neovascularization in response to test substances in polyacrylamide gels*

Test substance	Number of implants	Angiogenesis in rat bioassay*	Corneal vascular response†		
			Positive	Negative (%)	Inflammatory‡ (%)
Empty corneal pocket	12	—	—	12/12(100)	—
Controls:					
Polyacrylamide gel	18	Inactive	—	14/18(78)	4/18(22)
Tumor cells, nuclear, histone fraction	7	Inactive	—	6/7 (86)	1/7 (14)
Liver cells, nuclear, nonhistone fraction	4	Inactive	—	3/4 (75)	1/4 (25)
TAF:					
Cytoplasmic fraction	7	Active	5/7(72%)	1/7 (14)	1/7 (14)
Nuclear nonhistone fraction	5	Active	4/5(80%)	1/5 (20)	—

*As described by Folkman et al. (8).
†*See* text for criteria.
‡The incidence of inflammation was considerably reduced when Ringer's lactate was substituted for distilled water as the solvent for polyacrylamide reagents (*see* text under "Materials and Methods").

Fragments of homologous rabbit tumors (Brown-Pearce epithelioma and V2 carcinoma) exhibited 2 distinct phases of growth after corneal implantation: prevascular and vascular (text-fig. 2).

Central necrosis and peripheral localization of ^{3}H-TDR labeling (figs. 2, 3), within slowly growing, prevascular tumor plates, suggest that diffusion limitations for metabolites are a determining factor in their growth rate and geometry. Penetration and perfusion by corneal vessels (vascular phase) apparently provided a more efficient mode of metabolite exchange and were associated with transition to a more rapid, 3-dimensional growth pattern (text-fig. 2, figs. 7a and b, 8).

A similar contrast in tumor growth patterns before and after vascularization has also been observed in the anterior chamber of the rabbit eye (*5*). In that setting, the beginning of exponential growth of a tumor implant coincided with its vascularization from the subjacent iris. Before host vessels penetrated, growth rate for a given tumor was essentially linear. Prevention of any vascularization resulted in small, dormant tumors which remained viable but failed to grow. Therefore, as seen in situations where prevascular tumor growth can be directly observed, penetration by host vessels appears necessary for a rapid, malignant growth pattern.

The cornea before its vascularization behaves as an "immunologically privileged site" for homografts of corneal tissue (*11*). The current experiments suggest this is also so for tumor implants. Actual penetration of corneal vessels into xenografts, or incompatible homografts, was required for tumor rejection. Primary Brown-Pearce tumors never showed signs of rejection until 5–7 days after their vascularization, regardless of the duration of their prevascular growth. This apparent failure of sensitization by prevascular tumors may be related to the absence of lymphatic channels in the unvascularized cornea (*12, 13*).

All new blood vessel formation observed in these studies originated in surrounding host tissues. Although remnants of blood-filled vessels were occasionally visible within freshly implanted tumor fragments, outgrowth of these intrinsic capillaries did not occur. These findings agree with those of Merwin and Algire (*14*) who emphasized the host origin of new vessels stimulated by subcutaneous tumor grafts.

Corneal vessels were not formed in response to sham procedures, heat-killed tumor fragments, or necrotic tumor fragments. Viable implants stimulated capillary outgrowth which became organized into networks within the tumor parenchyma (fig. 8). In growing V2 carcinomas, these networks became progressively denser.

In regressing Brown-Pearce tumors, tertiary and secondary vascular branches rapidly disappeared, leaving a skeleton of hairpin channels (fig. 10). During the peak of regression, when tumor cell destruction was extensive (figs. 11, 12), no new corneal vessels were formed. Therefore, the presence of viable tumor cells appeared necessary for the formation and maintenance of corneal vessels.

The following observations suggest that this tumor-induced vascularization was mediated by diffusible substances: 1) When host vessels began growing, they were separated from the tumor implant by 1–3 mm of normal-appearing corneal stroma; 2) capillary budding always originated from a limited sector of the adjacent limbal plexus and developed into elongated hairpin loops directed toward the tumor edge; and 3) no capillaries formed until the border of the central tumors had grown within 2.5±0.5 (mean±SD, 10 implants) of the limbus, which often took 3–4 weeks. In contrast, peripheral implants (1–2 mm from limbus) elicited vessel formation as early as the 3d–4th day.

This phenomenon of a "critical distance" for initiation of vascularization and the directional outgrowth of the newly formed vessels are consistent with the release of a stimulatory factor(s) from the tumor site and its gradual diffusion through the corneal stroma to reach host vessels at the limbus (*15, 16*). Implants within the "critical distance" apparently provide a suprathreshold concentration to nearby limbal vessels, whereas central tumors must grow within this range before a threshold concentration is reached.

Further evidence for the diffusion of an angiogenic factor from intraocular tumors into surrounding host tissues was provided in (*9*), which documented neovascularization in the iris [illegible]

corneal tumors. Iris neovascularization also indicated that vascularization of the cornea is not merely a phenomenon peculiar to that tissue.

Neovascularization is a component of many disease processes of the cornea, and various theories have been proposed to explain this (*17*). Most recent workers, agreeing that corneal edema, per se, is not a sufficient stimulus for new vessel formation, have emphasized the role of chemical mediators (*16*, *18*). Intracorneal infusion of vasoactive or inflammatory amines, such as serotonin, bradykinin, and histamine, can produce vascularization (*19*), as does necrotizing trauma to the corneal stroma (*15*, *16*). In the current study, capillary outgrowth after viable tumor implants occurred without gross corneal edema or histologically significant stromal cell necrosis. Leukocytic infiltration was often minimal or absent, both in limbal vessels in the prevascular phase (fig. 4) and in outgrowing capillaries as they approached the tumor edge. However, we did not perform a detailed histologic analysis of the relationship between neovascularization and leukocytic infiltration or experiments to examine the effect of inhibition of leukocyte infiltration on capillary outgrowth.

Studies in this laboratory have led to the isolation of cytoplasmic and nuclear fractions from tumor cells (TAF), which are mitogenic for capillary endothelial cells (*3*, *6*) and can evoke neovascularization in test animals of other species (*1*). To help characterize the specificity of the tumor-induced corneal vascularization observed, a variety of active TAF fractions and inactive control preparations were tested for corneal vascularization activity.

The simple presence of new corneal vessels was not a satisfactory endpoint because inflammation with vascular pannus formation occurred with control polyacrylamide gels (fig. 18). However, daily observations with further criteria— 1) absence of gross corneal edema, 2) absence of pyogenic infiltrate, and 3) directional ingrowth and development of an organized capillary network within the area of the pocket— permitted differentiation of positive (figs. 16, 17) from negative (figs. 14, 15) or inflammatory patterns. As table 1 shows, positive neovascular patterns were produced only by active TAF fractions. Although the observations were few, the specificity of TAF-induced corneal vascularization and its similarity to the neovascular patterns elicited by tumor implants suggest a mechanism common to both, and further, tumor angiogenesis in the cornea may be comparable to that observed in other anatomic sites. Corneal gel implantation may, therefore, provide a model for the study of possible mediators of neovascularization in tumor growth and other pathologic states.

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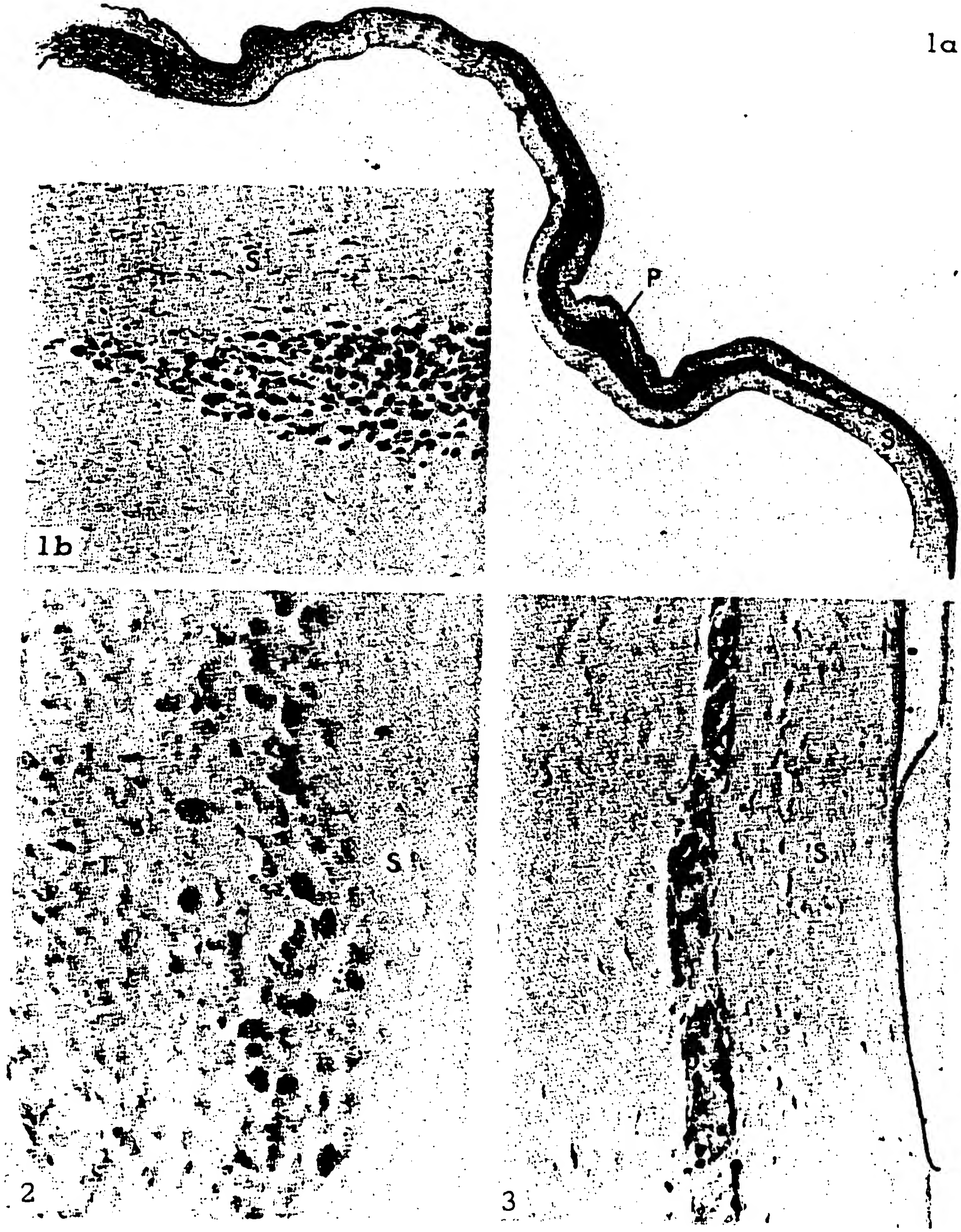


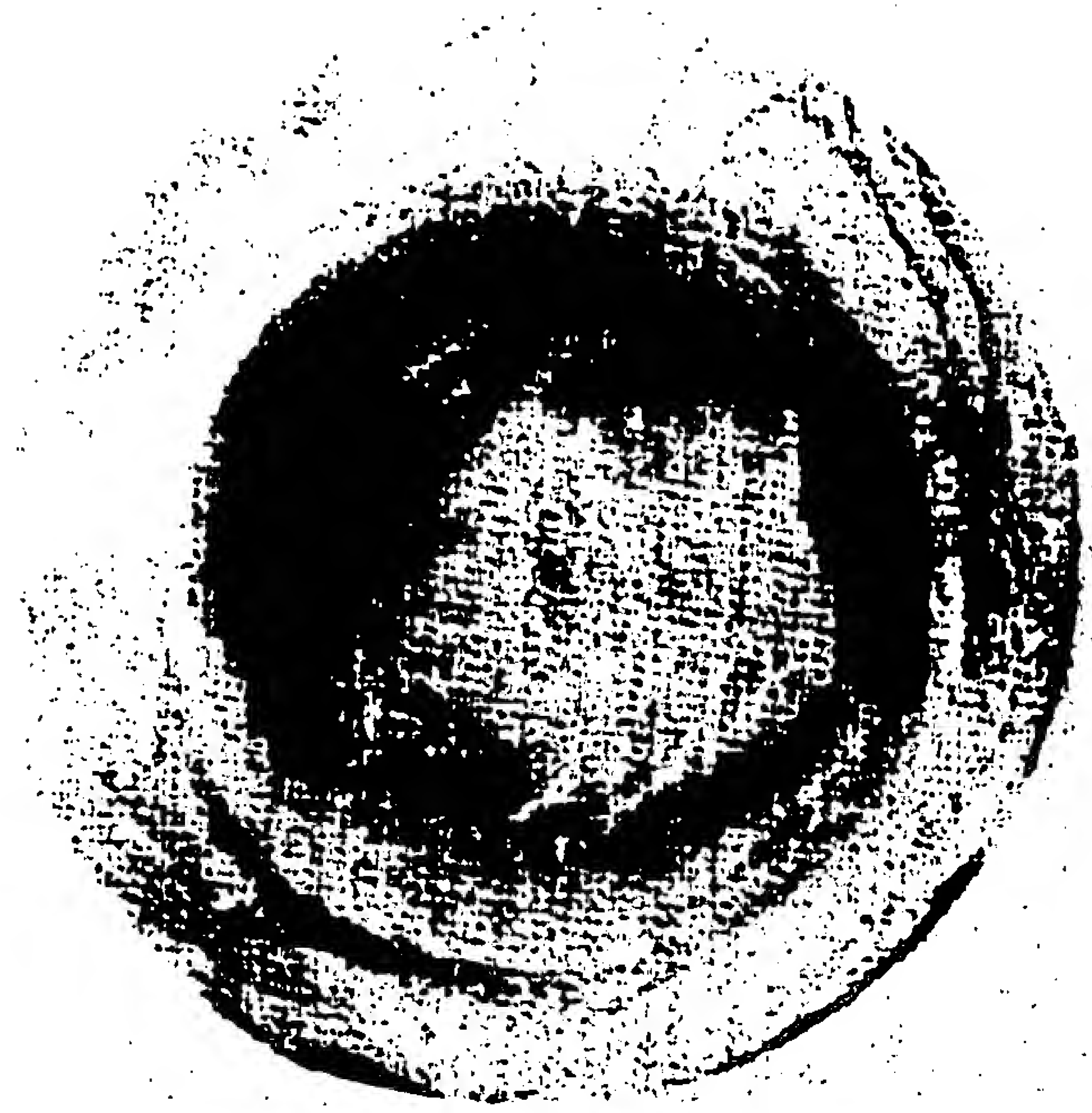
FIGURE 1.—*a*) Sagittal section of cornea 35 days after central implantation of a small fragment of Brown-Pearce epithelioma. Slowly growing avascular plate (P) of tumor cells remains sharply demarcated from surrounding corneal stroma (S). L = Limbus. Hematoxylin and eosin (H & E), × 10. *b*) *Note* normal appearance of corneal stroma (S) near tumor edge. × 150
FIGURE 2.—Autoradiograph of thicker portion of prevascular tumor (T) shown in figure 1, after in vitro exposure to ^{3}H-TDR. *Note* density of labeled tumor cells near periphery of plate. S = corneal stroma. × 300
FIGURE 3.—Autoradiograph of thin, growing edge of V2 carcinoma implant, after in vitro exposure to ^{3}H-TDR. *Note* many labeled tumor cells and absence of leukocytic infiltrate in surrounding corneal stroma (S). × 200

FIGURE 4.—Histologic section of limbal vessels from same cornea as shown in figure 1, in prevascular phase of tumor growth. *Note* absence of leukocytic infiltrate. H & E. × 180

FIGURE 5.—Survey photograph of colloidal carbon preparation showing whole cornea 27 days after central implantation of Brown-Pearce epithelioma. Tumor first grew as an avascular plate *(gray area)*. New vessel outgrowth *(black area*, enlarged in fig. 6) began when right-hand border of tumor approached within 2.5 mm of limbus. × 4

FIGURE 6.—Higher magnification of vascularized region of tumor plate shown in figure 5 (glycerin-cleared preparation). Extensive vascular network is typical of growing Brown-Pearce nodule (cf. with fig. 10 from regressing tumor). × 25

FIGURE 7.—*a*) Sagittal section of cornea 38 days after implantation of Brown-Pearce epithelioma fragment 3 mm from limbus. *Note* avascular tumor plate (P) and vascularized nodule (N) at its periphery. H & E. × 10. *b*) Higher magnification of vascularized nodule. *Arrow* points to early perivascular infiltrate. × 65



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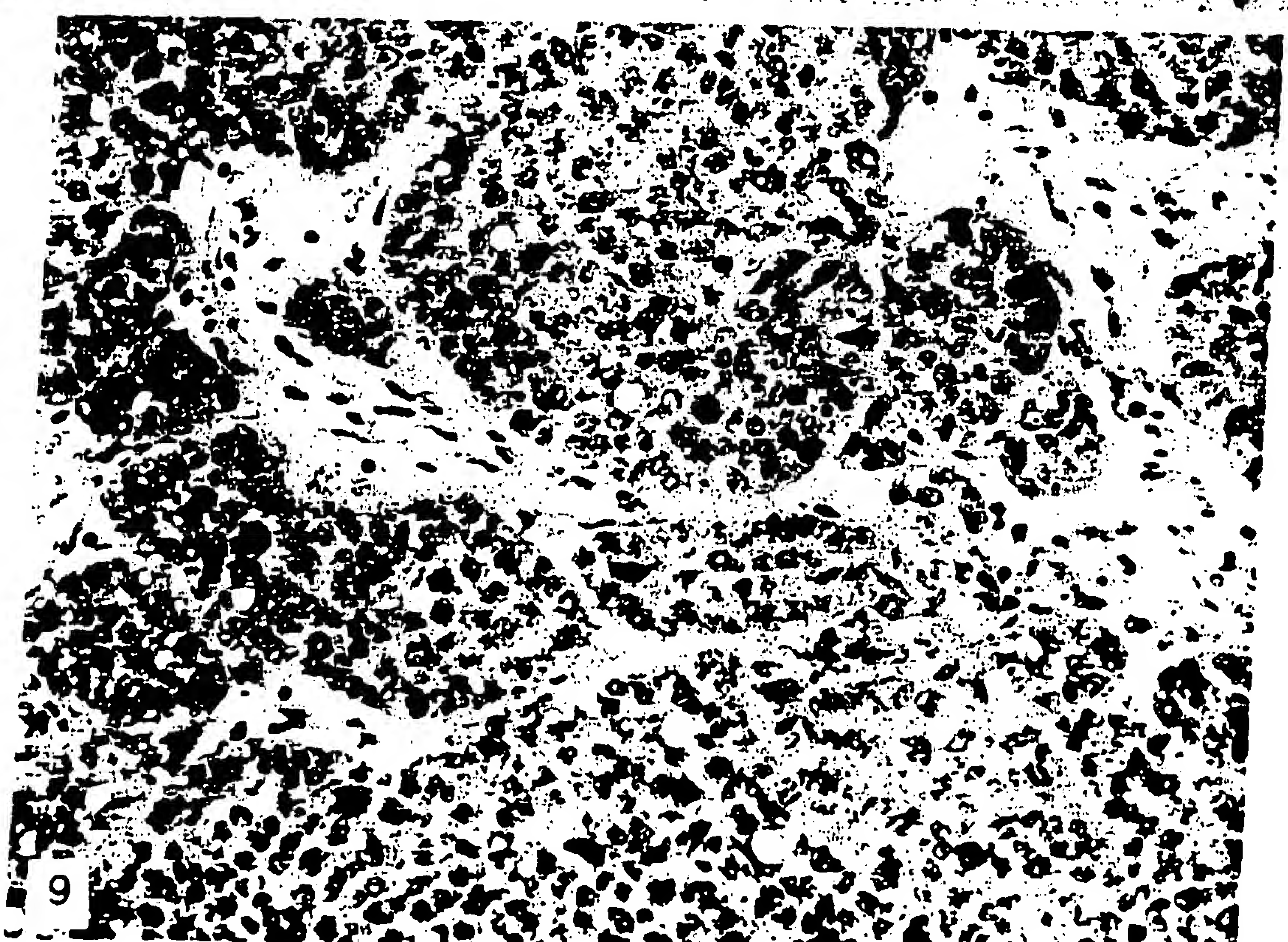


FIGURE 8.—Sagittal section of cornea 19 days after implantation of V2 carcinoma fragment 2 mm from limbus. Within 10 days after its vascularization, this tumor grew into an exophytic mass measuring $1.2 \times 1.0 \times 0.6$ mm^3. H & E. $\times 10$

FIGURE 9.—Higher magnification of portion of V2 carcinoma shown in figure 8. *Note* anaplastic tumor parenchyma and richly vascularized stroma. $\times 300$

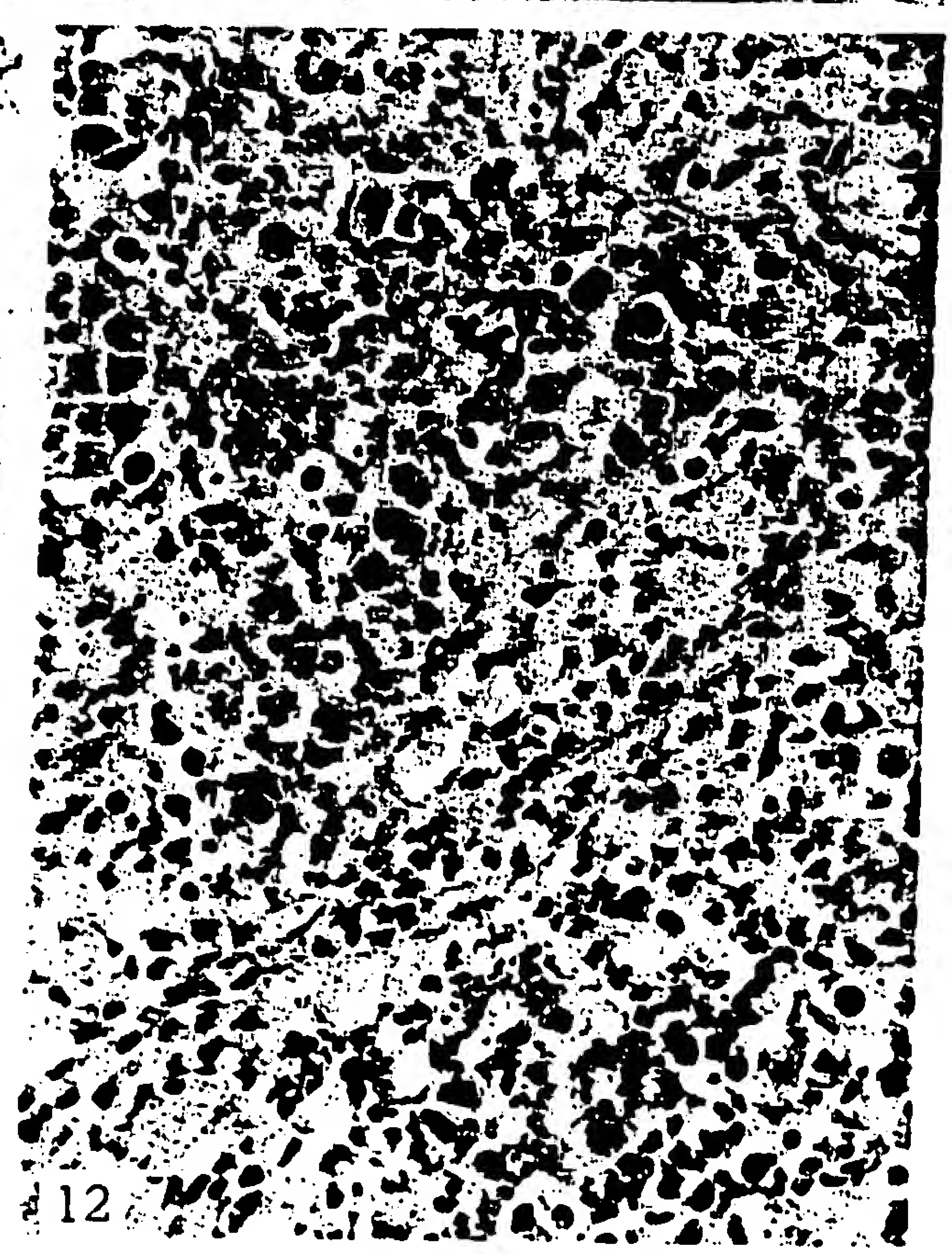


FIGURE 10.—Colloidal carbon injection of corneal vessels within Brown-Pearce nodule during advanced stage of regression. Compare "pruned-tree" appearance of this network with more extensive arborization seen in growing tumors (fig. 6). Glycerin-cleared wholemount preparation. × 14

FIGURE 11.—Histologic section of Brown-Pearce nodule during advanced stage of regression. Diffuse and nodular (arrow) lymphocytic infiltrate. Compare edematous, less compact appearance of this tumor to early regressing tumor shown in figure 7b. H & E. × 65

FIGURE 12.—Higher magnification of regressing Brown-Pearce nodule showing cytoplasmic vacuolization, nuclear fragmentation, and pyknosis. H & E. × 300

Figures 13–18: Low-power micrographs of colloidal carbon-injected, glycerin-cleared preparations of corneas containing polyacrylamide gel implants. P = site of pocket. Days refer to time after implantation. "Active" and "inactive" refer to results in rat bioassay (table 1).

FIGURE 13.—Saline-gel control (29 days); completely negative response.

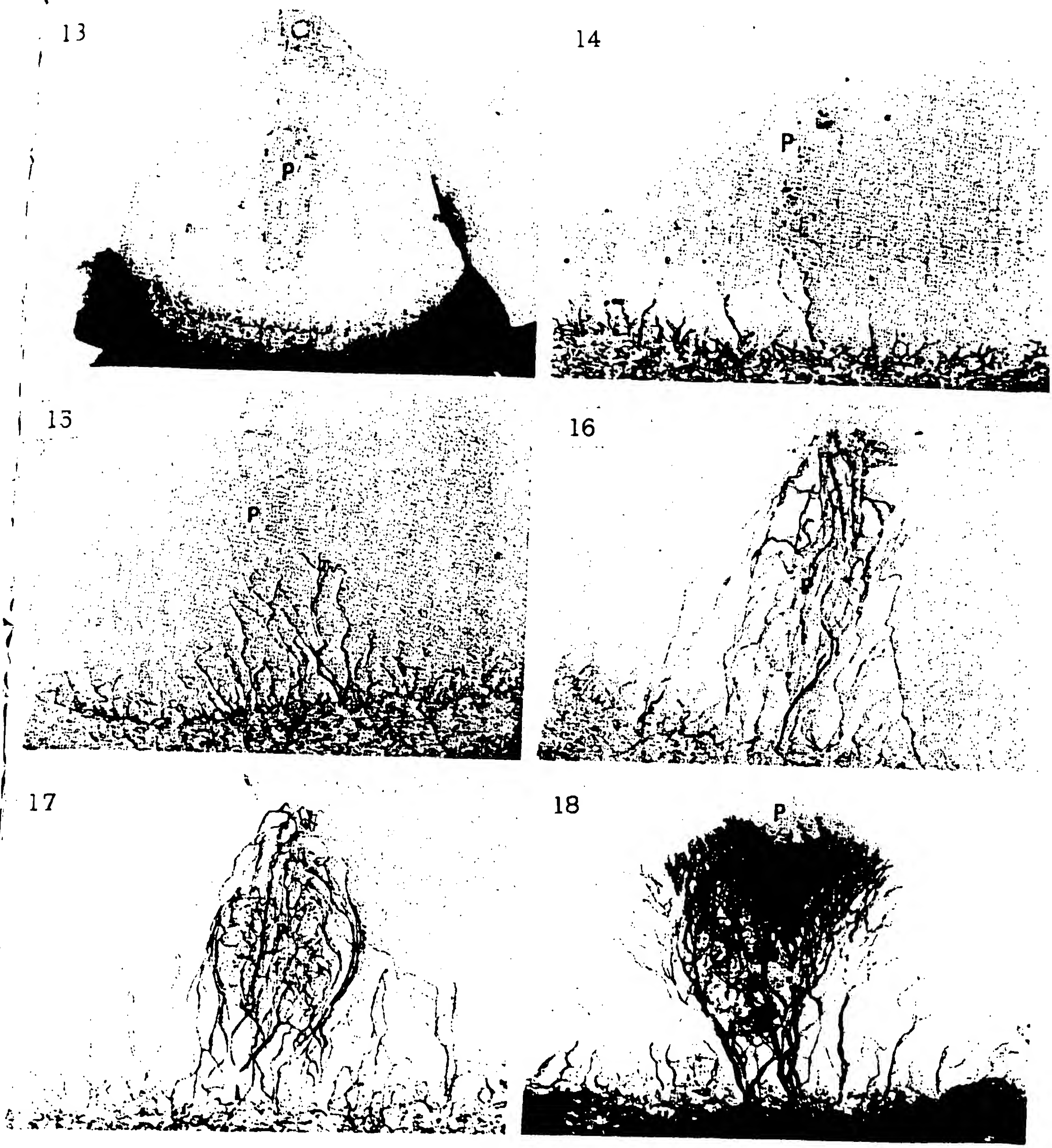
FIGURE 14.—Saline-gel control (22 days); negative response.

FIGURE 15.—TAF-inactive, nuclear, histone fraction from Walker 256 tumor cells (22 days); negative response. A few vessels reach but do not penetrate pocket area.

FIGURE 16.—TAF-active, nuclear, nonhistone fraction from Walker 256 tumor cells (22 days); positive response. Abundant vascularization developed after 7 days and arborized throughout pocket area.

FIGURE 17.—TAF-active, cytoplasmic fraction from Walker 256 tumor cells (31 days); positive response. Vessel network grew into pocket area at end of 1st week and persisted until animal was killed 3 weeks later. No gross corneal edema or pyogenic infiltrate was observed.

FIGURE 18.—TAF-inactive, nuclear, histone fraction from Walker 256 tumor cells (22 days); inflammatory response. Conjunctivitis, iritis, gross corneal edema, and pyogenic infiltration of pocket occurred within the first 3–5 days and were followed by development of a dense, vascular pannus during the 2d week.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Medical Sciences

Thalidomide is an inhibitor of angiogenesis

(fibroblast growth factor/rabbit cornea)

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ABSTRACT Thalidomide is a potent teratogen causing dysmelia (stunted limb growth) in humans. We have demonstrated that orally administered thalidomide is an inhibitor of angiogenesis induced by basic fibroblast growth factor in a rabbit cornea micropocket assay. Experiments including the analysis of thalidomide analogs revealed that the antiangiogenic activity correlated with the teratogenicity but not with the sedative or the mild immunosuppressive properties of thalidomide. Electron microscopic examination of the corneal neovascularization of thalidomide-treated rabbits revealed specific ultrastructural changes similar to those seen in the deformed limb bud vasculature of thalidomide-treated embryos. These experiments shed light on the mechanism of thalidomide's teratogenicity and hold promise for the potential use of thalidomide as an orally administered drug for the treatment of many diverse diseases dependent on angiogenesis.

Thalidomide is a potent teratogen. It was developed by Chemie Grunenthal in the 1950s as a sedative that appeared so nontoxic in rodent models that a LD_{50} could not be established. In 1961, McBride (1) and Lenz (2) described the association between limb defects in babies and maternal thalidomide usage. Although humans are exquisitely sensitive to the teratogenic effects of thalidomide, experiments in rodents failed to reveal similar effects (3, 4). Teratogenic effects could be experimentally reproduced by the administration of thalidomide to pregnant rabbits at an oral dose of 100–300 mg per kg per day (5, 6). Over the past 30 years, the mechanism of thalidomide's teratogenicity has been extensively studied but has remained unsolved (7).

We now postulate that the limb defects seen with thalidomide were secondary to an inhibition of blood vessel growth in the developing fetal limb bud. The limb bud is unique in requiring a complex interaction of both angiogenesis and vasculogenesis during development (8). Vasculogenesis is the formation of a capillary bed from endothelial cells that have differentiated from mesenchymal precursors. Angiogenesis is the formation of new blood vessels from sprouts of preexisting vessels. Therefore, the limb bud would be a particularly vulnerable target to a teratogen that inhibited endothelial cell function. We chose to examine the effect of thalidomide on growing vasculature in the chicken chorioallantoic membrane and in the rabbit cornea.

MATERIALS AND METHODS

Chicken chorioallantoic membrane (CAM) assays were performed as described (9, 10) and the effects on the developing vasculature were recorded at 48 h after implantation of the 0.5% carboxymethylcellulose pellet containing various drugs. Corneal neovascularization was induced by an implanted pellet of poly(hydroxyethyl methacrylate) (Hydron; Interferon Sciences, New Brunswick, NJ) containing 650 ng of the potent angiogenic protein basic fibroblast growth factor (bFGF) bound to sucralfate (sucrose aluminum sulfate; Bukh Meditec, Copenhagen) (11). The addition of sucralfate to the pellet protects the bFGF from degradation (12) and provides for its slow release, thus producing consistent aggressive angiogenesis that is more pronounced than that induced by bFGF/Hydron alone. Release of bFGF from pellets containing sucralfate/Hydron could be detected *in vitro* for up to 4 days after the pellets were formed compared to just 1 day for pellets with Hydron alone (11). Pellets were made by mixing 110 μl of saline containing 12 μg of recombinant bFGF (Takeda, Osaka) with 40 mg of sucralfate; this suspension was added to 80 μl of 12% (wt/vol) Hydron in ethanol. Aliquots (10 μl) of this mixture were then pipetted onto Teflon pegs and allowed to dry producing approximately 17 pellets. A pellet was implanted into corneal micropockets of each eye of an anesthetized female New Zealand White rabbit, 2 mm from the limbus, followed by a single topical application of erythromycin ointment on the surface of the cornea. Histologic examination on consecutive days demonstrated progressive blood vessel growth into the cornea toward the pellet with only rare inflammatory cells seen. This angiogenic response was not altered by severe immune suppression with total body irradiation, and pellets with sucralfate alone did not induce angiogenesis (data not shown). Unlike other models of corneal angiogenesis that utilize inflammation to stimulate neovascularization, the new vessels are primarily induced by the bFGF. The animals were fed daily from 2 days after implantation by gastric lavage with either drug suspended in 0.5% carboxymethylcellulose or vehicle alone. Thalidomide was purchased from Andrulus Pharmaceutical (Beltsville, MD) and EM-12 and Supidimide were kindly provided by Grunenthal (Stolberg, F.R.G.). Immunosuppressed animals received total body radiation of 6 Gy for 6 min immediately prior to implantation of the pellets. This dose of radiation resulted in a marked leukocytopenia with >80% reduction in the leukocyte count by day 2 and >90% reduction by day 3, results that are consistent with previous reports (13, 14).

The animals were examined with a slit lamp every other day in a masked manner by the same corneal specialist (M.S.L.). The area of corneal neovascularization was determined by measuring with a reticule the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. A formula was used to determine the area of a circular band segment: $C/12 \times 3.1416\,[r^2 - (r - L)^2]$, where $r = 6$ mm, the measured radius of the rabbit cornea. We have utilized various mathematical models to determine the amount of vascularized cornea and have found that this formula provides the most accurate approximation of the area of the band of neovascularization that grows toward the pellet. Only the uniform contiguous band of neovasculariza-

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Abbreviations: bFGF, basic fibroblast growth factor; CAM, chicken chorioallantoic membrane; PGA, phthaloylglutamic anhydride; PG acid, phthaloylglutamic acid; TNF-α, tumor necrosis factor α.

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tion adjacent to the pellet was measured. The noncontiguous neovascularization, which can be seen superiorly, was not quantified due to its irregular shape. These vessels that often grow concurrently toward the pellet from the superior limbus arise from vessels of the superior rectus supplying the limbus, are directly induced by the bFGF/sucralfate pellet, and are histologically identical to the inferior limbal vessels. However, it should be noted that this superior neovascularization was commonly seen in control animals and was never seen in thalidomide-treated animals. Thus, the total inhibition of neovascularization is conservatively underestimated.

RESULTS

Our initial investigations were performed on the CAM. Neither thalidomide nor EM-12, a related teratogenic analog (15), exhibited any inhibitory activity on blood vessel growth. This result was expected as it has been proposed that thalidomide must be metabolized by the liver to form an epoxide that may be the active teratogenic metabolite (16). Other thalidomide analogs that have been shown to be teratogenic in rodents (17), including phthaloylglutamic anhydride (PGA) and phthaloylglutamic acid (PG acid), were also analyzed (Fig. 1). Interestingly, weak antiangiogenic activity of the developing vasculature was seen with both PG acid and PGA when 100 μg of either compound was placed on the CAM in a pellet of 0.5% carboxymethylcellulose. Despite frequent mild scarring, avascular zones were produced in 15% of the CAMs with PGA compared to control 0.5% carboxymethylcellulose pellets in which no avascular zones were seen (data not shown).

Based on these initial findings, we decided to test thalidomide's effect on angiogenesis induced by bFGF in the rabbit corneal micropocket model. Treatment with a teratogenic dose (200 mg/kg) of thalidomide resulted in an inhibition of the area of vascularized cornea that ranged from 30 to 51% in three experiments with a median inhibition of 36% (Figs. 2*A* and 3) (n = 30 eyes; P = 0.0001, two-way ANOVA with ranked data). The inhibition of angiogenesis by thalidomide was seen after only two doses (Fig. 2*B*). The rabbits did not demonstrate obvious sedation and there were no signs of toxicity or weight loss. The teratogenic analog EM-12, which shares the other properties of thalidomide, was also inhibitory, with a median inhibition of 42% (n = 10 eyes; P = 0.002, one-way ANOVA with ranked data). Supidimide, a nonteratogenic analog that retains the sedative properties of thalidomide, exhibited no activity (area 107% of control; n = 10 eyes; not statistically different from control). Other analogs, PGA and PG acid, displayed weaker inhibitory effects

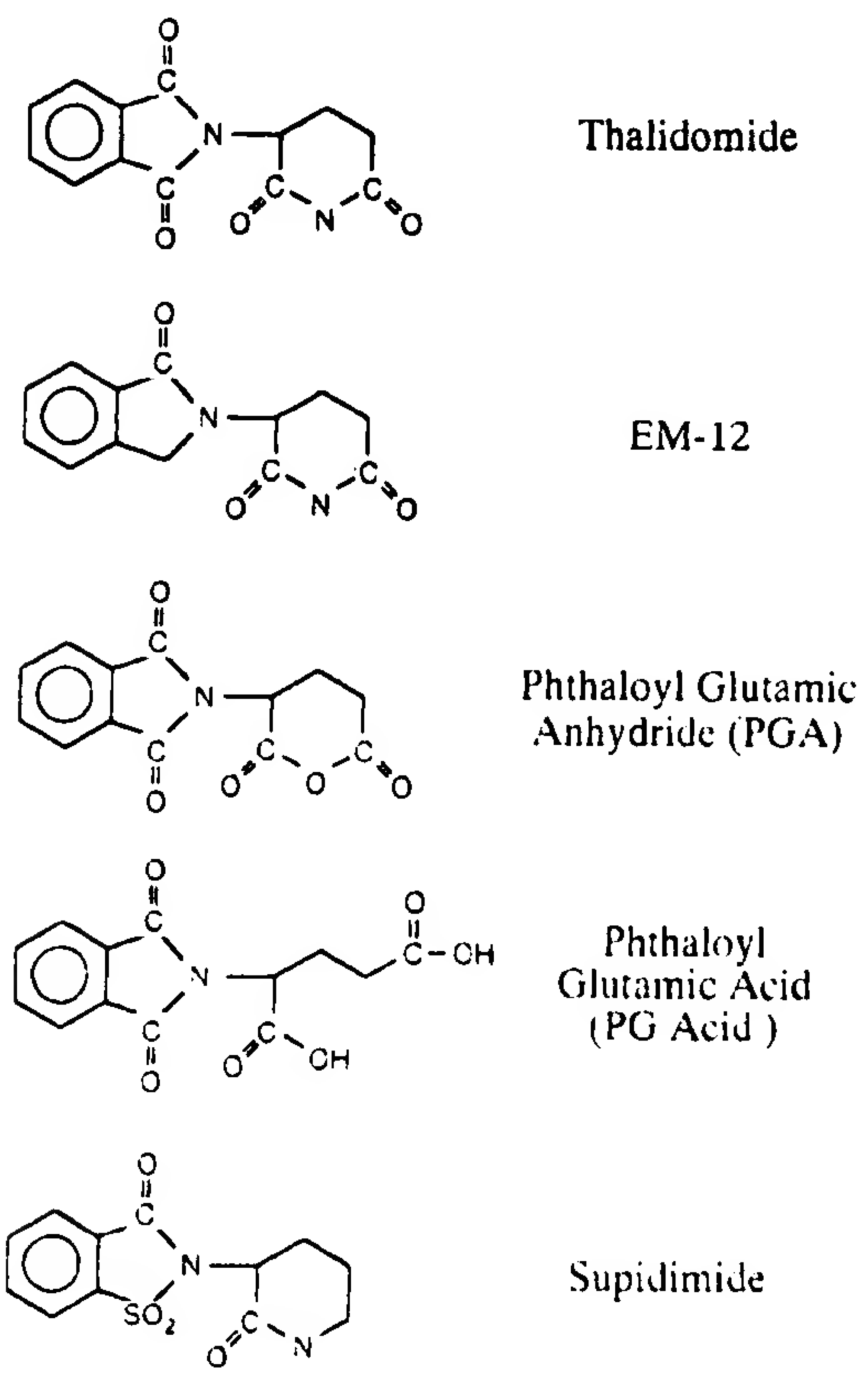

FIG. 1. Structure of thalidomide and related analogs.

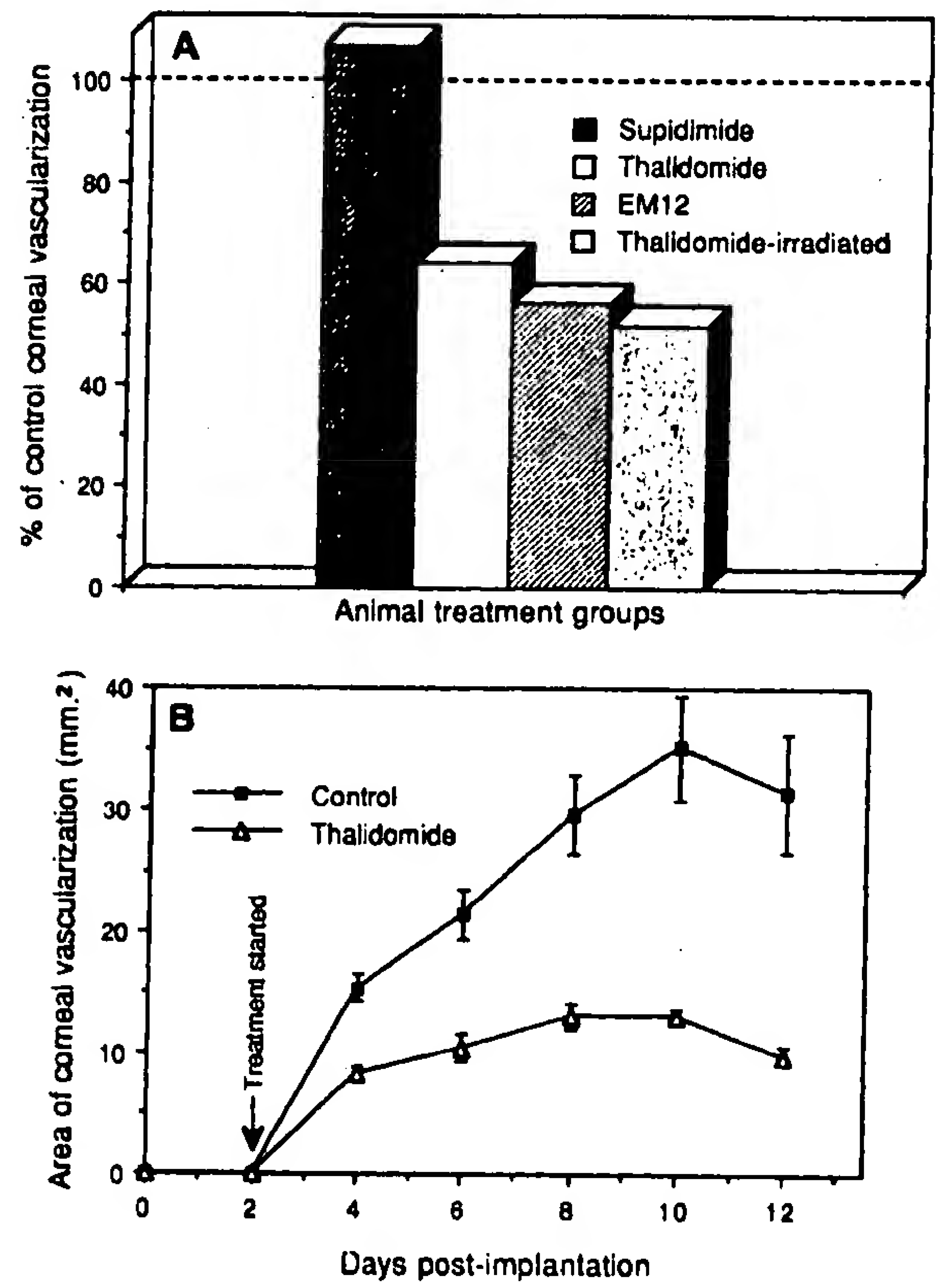

FIG. 2. (*A*) Inhibition of bFGF-induced corneal neovascularization by thalidomide and related analogs expressed as percent of median control on day 8. Pellets containing bFGF and sucralfate were implanted into micropockets of both corneas of rabbits (18). Vessel ingrowth into the clear cornea from the limbus was first noted on day 2 and treatments (200 mg/kg orally) were begun on this day. The area of corneal neovascularization was measured from day 4 through day 12. Day 8 measurements were used for comparison between groups. No regression of vessels and near maximal neovascularization was seen at this time point. Statistical analysis was performed with ANOVA with ranked data to account for interexperimental variation and to guard against a nonnormal distribution of data (i.e., outliers) by utilizing a nonparametric method. (*b*) Time course of inhibition of neovascularization with thalidomide. Mean areas of corneal neovascularization with standard error bars are presented from one experiment that is representative of the three experiments performed with thalidomide on nonirradiated animals. Data presented from the first time point after administration of the drug through the completion of the study are statistically different (n = 10 eyes; P < 0.005 for all time points, one-way ANOVA with ranked data).

than thalidomide (data not shown). The density of vessel ingrowth in thalidomide-treated animals was also markedly reduced. Due to the lack of an objective grading scale, these results are not presented.

Thalidomide has immunosuppressive properties that might have indirectly affected angiogenesis. Recently, thalidomide has been used for its immunosuppressive properties in the treatment of leproma reactions (19) and chronic graft versus host disease (18, 20–23). However, in humans its immunosuppressive properties are weak and delayed with little effect in acute graft versus host disease (24). Because the effect of thalidomide on the immune system is similar but weaker than that of cyclosporin A (25), we tested cyclosporin A at the highest tolerated dose of 25 mg/kg. No statistically significant effect was observed compared to control. To investigate further the immune interactions, we pretreated the rabbits with the maximally tolerated immunosuppressive dose of total body irradiation. Immunosuppressed animals responded equally well to thalidomide, with a median inhibition of neovascularization of 52% (n = 12; P = 0.0001, one-way ANOVA with ranked data) as compared to irradiated placebo-treated controls (Fig. 2*A*).

Electron microscopic examination of corneas from thalidomide-treated and control animals revealed ultrastructural differences. Vessels in the thalidomide-treated group dem-

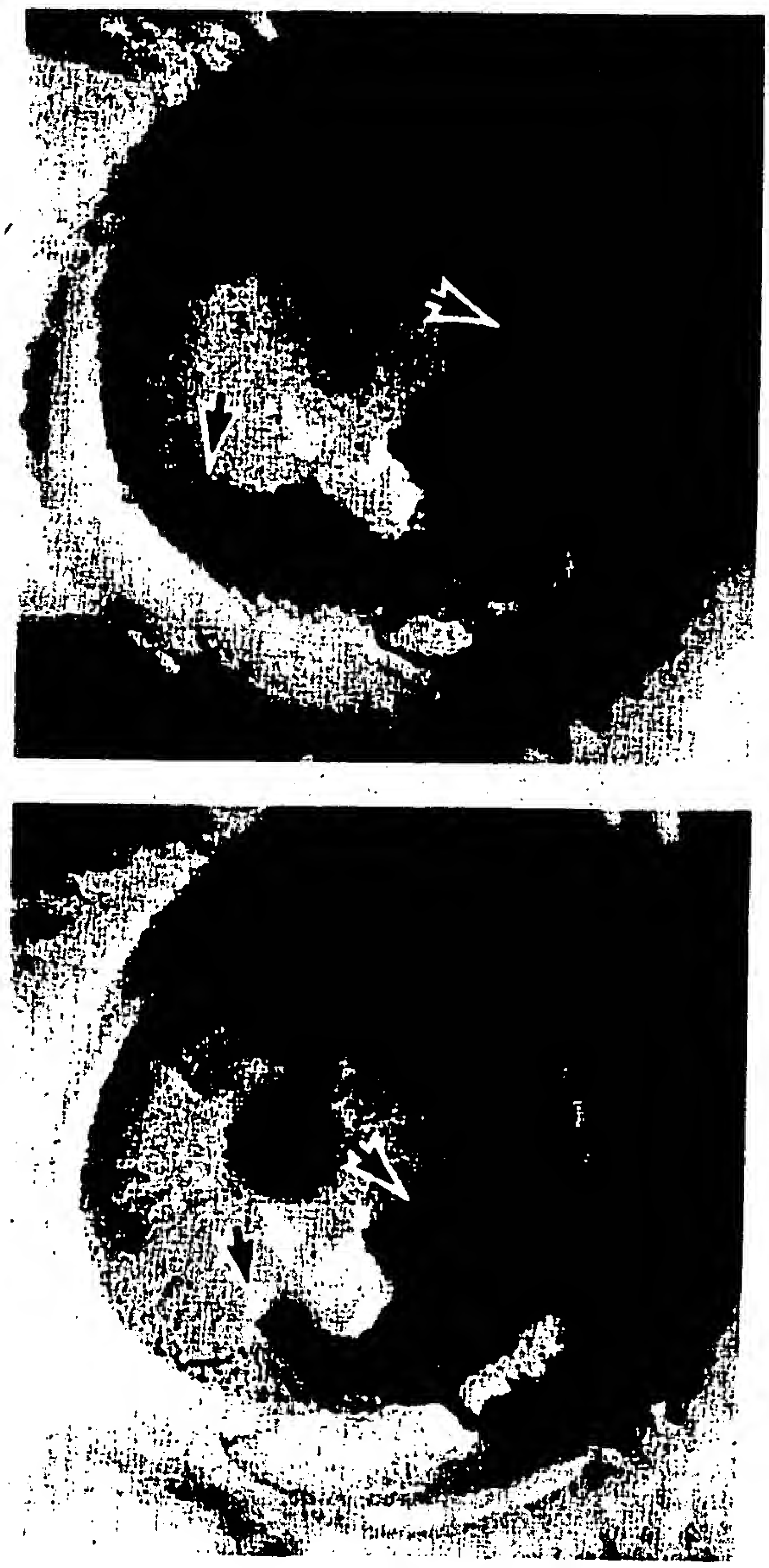

FIG. 3. Representative corneas at 8 days after implantation of bFGF pellets from control (*A*) and thalidomide-treated (*B*) rabbits. There is prominent corneal neovascularization (arrows) in the control with associated corneal clouding, which was demonstrated histologically to be stromal edema without inflammation. The thalidomide-treated animal has markedly less neovascularization with minimal corneal edema.

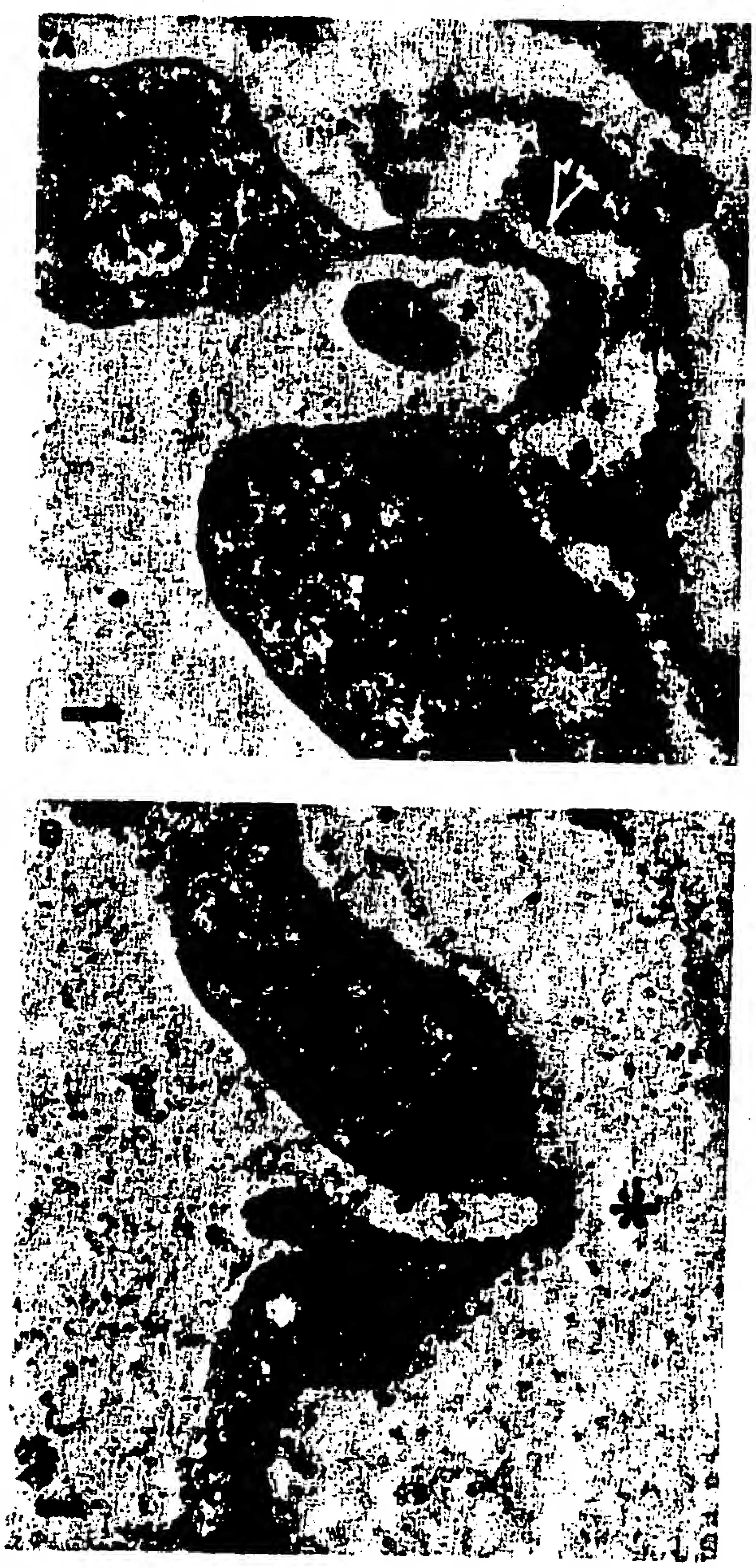

FIG. 4. Electron micrographs of corneal neovascularization observed in a thalidomide-treated rabbit 10 days after implantation of a pellet containing bFGF. (*A*) High-magnification (×40,000) view of typical fenestrations (arrow) in an endothelial cell from corneal neovascularization in thalidomide-treated rabbit. (*B*) High-magnification (×60,000) view of an area of cell thinning (asterisk) adjacent to a cell junction in thalidomide-treated corneal neovascularization. These changes were not seen in control day 10 corneal neovascularization. (Bars = 0.1 μm.)

onstrated fenestrations not seen in control animals (Fig. 4*A*). Fenestrations have been previously reported to be specific to regressing corneal blood vessels after removal of the angiogenic stimulus (26). However, in that model, endothelial cell regression was associated with platelet plugging and cellular hypoxic changes such as swollen mitochondria, which were not seen in the thalidomide-treated animals. Interestingly, histologic changes previously described in the vasculature of the limb buds from chicken embryos treated with thalidomide (27) were also seen in the corneal neovascularization of our thalidomide-treated rabbits including vesicular projections into the lumen and extreme thinning of cell processes (Fig. 4*B*). In general, the corneal neovascularization from thalidomide-treated rabbits appeared more immature than that observed in control animals with poorly formed cell junctions, incomplete basement membrane, and fewer associated pericytes. These findings support the hypothesis that thalidomide has a direct effect on the growing vasculature.

DISCUSSION

Orally administered thalidomide is an inhibitor of angiogenesis induced by bFGF in the rabbit cornea micropocket assay. The mechanism by which thalidomide inhibits angiogenesis is unknown. Thalidomide has shown no effect on bFGF-induced proliferation of endothelial cells in culture (data not shown). Current studies are focused on the identification of the most active thalidomide metabolite. The formation of an active metabolite by the liver *in vivo* provides an explanation of the observation that the effect of thalidomide on growing vessels is seen only when given systemically.

Thalidomide has been shown to suppress tumor necrosis factor α (TNF-α) production from macrophages (28). However, macrophages were rarely seen in histologic examinations of our model of corneal neovascularization. Furthermore, studies examining the role of TNF-α in corneal angiogenesis have failed to detect TNF-α production in a model of inflammatory corneal angiogenesis in which macrophages were prominent (29). TNF-α is only weakly angiogenic *in vivo*. It acts by inducing secondary inflammation in contrast to bFGF, which stimulates angiogenesis without inflammation (30). Thus, the ability of thalidomide to inhibit angiogenesis induced by pharmacologic doses of bFGF supports the hypothesis that thalidomide directly inhibits an essential component of angiogenesis and does not operate through effects on TNF-α production.

In conclusion, thalidomide is a potent angiogenesis inhibitor *in vivo*. In this model of corneal angiogenesis, we have tested many putative angiogenesis inhibitors (including antimitotic agents, *cis*-retinoic acid, tamoxifen, and others). Thalidomide was the only agent capable of inhibiting angiogenesis after oral administration. Evaluation of thalidomide analogs demonstrated a correlation between teratogenicity and antiangiogenic potential. The weak and delayed immunosuppressive action of thalidomide when used clinically, its inhibition of angiogenesis in radiation-immunosuppressed animals, and the lack of effect of the functionally related immunosuppressive agent cyclosporin A argue for a direct effect of thalidomide on angiogenesis. Further support for this hypothesis is derived from the ultrastructural changes seen in thalidomide-treated animals. There are clear implications for the use of this drug in the treatment of pathologic angiogenesis that occurs in diabetic retinopathy, macular degeneration, and solid tumors. Because antiangiogenic therapy is likely to be long-term, there is a need for an orally efficacious inhibitor.

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